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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A01N 43/04, A61K 31/70, A01N 63/00, A61K 48/00, C07H 21/04, C12N 15/09, 15/63, 15/85, 5/10, 9/06		A1	(11) International Publication Number: WO 96/00006 (43) International Publication Date: 4 January 1996 (04.01.96)
(21) International Application Number: PCT/US95/07849 (22) International Filing Date: 20 June 1995 (20.06.95) (30) Priority Data: 08/265,046 24 June 1994 (24.06.94) US (71) Applicant: UNIVERSITY OF PITTSBURGH OF THE COMMONWEALTH SYSTEM OF HIGHER EDUCATION [US/US]; 911 William Pitt Union, Pittsburgh, PA 15260 (US). (72) Inventors: BILLIAR, Timothy, R.; 5524 Fair Oaks Street, Pittsburgh, PA 15217 (US). TZENG, Edith; 1 Triamont Lane #502P, Pittsburgh, PA 15211 (US). NUSSLER, Andreas, K.; Hartweg 4, D-89231 Neu-Ulm (DE). GELLER, David, A.; 2329 Hollywood Drive, Pittsburgh, PA 15235 (US). SIMMONS, Richard, L.; 123 S. Murtland Avenue, Pittsburgh, PA 15208 (US). (74) Agent: GOULD, Lewis, F.; Eckert Seamans Cherin & Mellott, Suite 3232, 1700 Market Street, Philadelphia, PA 19103 (US).		(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG). Published <i>With international search report.</i>	
(54) Title: INDUCIBLE NITRIC OXIDE SYNTHASE GENE FOR TREATMENT OF DISEASE (57) Abstract The present invention discloses a full-length human hepatocyte iNOS cDNA clone and various gene therapy applications utilizing an iNOS DNA sequence. In preferred embodiments of the disclosed invention, iNOS-directed gene therapy involves specific targeting of a DNA sequence encoding a protein or protein fragment with iNOS biological activity for treating vascular diseases and disorders, antitumor applications and in response to certain microbial infections.			

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INDUCIBLE NITRIC OXIDE SYNTHASE GENE FOR TREATMENT OF DISEASE

The invention described herein was made in the course of work supported in part by Public Health Service, Grant Nos. GM44100 and GM37753 from the United States National Institutes of Health, General Medical Sciences. The United States Government
5 has certain rights in this invention.

1. INTRODUCTION

This invention relates to a human tissue inducible nitric oxide synthase cDNA clone capable of expressing a human inducible nitric oxide synthase protein, and a process
10 suitable for cloning a cDNA encoding amino acid sequences for the human inducible nitric oxide synthase. More specifically, this invention relates to a human hepatocyte inducible nitric oxide synthase cDNA clone and to a process for cloning and expression of the human hepatocyte inducible nitric oxide synthase cDNA to provide a source of the human hepatocyte inducible nitric oxide synthase enzyme.

15 Figures 1A-G show the 4,145 nucleotide bases for the sense strand of cDNA for human hepatocyte inducible nitric oxide synthase and sets forth the base codes as triplets (codon) for the coding parts of the nucleotide sequence. Figures 1A-G show the amino acid sequence for the cDNA clone for human hepatocyte inducible nitric oxide synthase encoding amino acids 1 through 1153 of the human hepatocyte inducible nitric oxide
20 synthase enzyme.

The invention further relates to the use of a nucleic acid sequence encoding inducible NOS (iNOS) or a biologically active iNOS protein fragment in gene therapy applications of mammalian host diseases or disorders. Such maladies include, but are not limited to, treatment of vascular occlusive disease, as well as cancer, and microbial
25 infection.

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2. BACKGROUND OF THE INVENTION

It is known by those skilled in the art that nitric oxide (NO) is a biologic mediator derived from the amino acid L-arginine. A family of enzymes, known as nitric oxide synthase (NOS), act upon L-arginine to oxidize one of the guanidino nitrogens to nitric oxide while citrulline is formed from the remainder of the L-arginine molecule. Nitric oxide is a very short-lived free radical and is rapidly oxidized to nitrite (NO_2^-) and nitrate (NO_3^-) which are measured as the stable inactive end products of nitric oxide formation.

It is well known by those skilled in the art that multiple isoforms of the nitric oxide synthase enzyme exist and that they are generally classified into two broad categories: (1) constitutive and (2) inducible. These classes of NOS enzymes vary considerably in size, amino acid sequence, activity and regulation. For example, cells such as neurons and vascular endothelial cells contain constitutive NOS isoforms while macrophages and vascular smooth muscle cells express an inducible NOS.

It is generally well known that the small amounts of nitric oxide generated by a constitutive NOS appear to act as a messenger molecule by activating soluble guanylate cyclase and, thus, increasing intracellular guanosine, 3', 5' - cyclic monophosphate (cGMP) and the induction of biological responses that are dependent on cGMP as a secondary messenger. For example, through this mechanism, endothelium derived nitric oxide induces relaxation of vascular smooth muscle and is identified as endothelium derived relaxing factor (EDRF) [Palmer, et al., 1987, Nature 327: 524-526 and Ignarro, et al., 1987, Proc. Natl. Acad. Sci. USA 84: 9265-9269]. Another example includes, but is not limited by, neuronal nitric oxide which acts as a neurotransmitter by activating guanylate cyclase with important functions in the central nervous system and autonomic nervous system (Bredt, et al., 1989, Proc. Natl. Acad. Sci. USA 86: 9030-9033 and Burnett, et al., 1992, Science 257: 401-403). It is generally known by those skilled in the art that the sustained production of nitric oxide by the inducible nitric oxide synthase has antimicrobial and antitumor functions. (see Granger, et al., 1989, J. Clin. Invest. 81: 1129-1136 and Hibbs, et al., 1987, Science 235: 473-476, respectively). It is also known by those skilled in the art that when vascular smooth muscle cells are stimulated to express a NOS enzyme by inflammatory cytokines, the large amounts of nitric oxide released contribute to the vasodilation and hypotension seen in sepsis (Busse and Mulsch, 1990, FEBS Letter 265: 133-136).

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Thus, it will be appreciated that nitric oxide has normal physiologic intracellular and extracellular regulatory functions, and in some instances excessive production of nitric oxide can be detrimental. For example, stimulation of inducible nitric oxide synthesis in blood vessels by bacterial endotoxin, such as for example bacterial lipopolysaccharide (LPS), and cytokines that are elevated in sepsis results in excessive dilation of blood vessels and sustained hypotension commonly encountered with septic shock (Kilbourn, et al., 1990, Proc. Natl. Acad. Sci. USA 87: 3629-3632). It is known that overproduction of nitric oxide in lungs stimulated by immune complexes directly damages the lung (Mulligan, et al., 1992, J. Immunol. 148: 3086-3092). Induction of nitric oxide synthase in pancreatic islets impairs insulin secretion and contributes to the onset of juvenile diabetes (Corbett, et al., 1991, J. Biol. Chem. 266: 21351-21354). Production of nitric oxide in joints in immune-mediated arthritis contributes to joint destruction (McCartney, et al., 1993, J. Exp. Med. 178: 749-754).

It will be appreciated that there is a great need in the medical community for selective inhibition of the inducible form of NOS but not the constitutive types of NOS in humans because this would allow for a means of preventing, such as for example, the damage of pancreatic islets or joint destruction in arthritis without preventing the physiologic regulation of vasomotor tone or neurotransmission in the central nervous system.

In many situations nitric oxide even when produced in high amounts as seen with inducible nitric oxide synthase expression can be beneficial. For example, induced nitric oxide synthesis is important in preventing liver damage in endotoxemia (Billiar, et al., 1990, J. Leuk. Biol. 48: 565-569; Harbrecht et al, 1992, J. Leuk. Biol. 52:390-392).

Several references attempt to link nitric oxide to changes seen in vascular disease. For example, Bucala, et al. (1991, J. Clin. Inv. 87: 432-438) disclose that glycosylation products that accumulate in vessel walls during hyperglycemia may quench nitric oxide and reduce nitric oxide availability. Chin, et al. (1992, J. Clin. Inv. 89: 10-18) disclose that oxidized lipoproteins have a similar effect by inactivating nitric oxide. Chester, et al. (1990, Lancet 336: 897-900) disclose that nitric oxide synthesis is reduced in atherosclerotic epicardial arteries in humans. None of these references shed light on therapeutic avenues regarding iNOS-driven gene therapy.

Actions of nitric oxide important to vascular integrity and the prevention of the atherosclerotic lesion include vasodilation (Palmer, et al., 1987, *Nature* 327: 524-526; Ignarro, et al., 1987, *Proc. Natl. Acad. Sci. USA* 84: 9265-9269), inhibition of platelet adherence and aggregation (Radomski, et al., 1987, *Br. J. Pharmacol.* 92: 639-646),
5 inhibition of vascular smooth muscle (Nunokawa, et al., 1992, *Biochem. Biophys. Res. Com.* 188:409-415) and fibroblast (Werner-Felmayer, et al., 1990, *J. Exp. Med.* 172: 1599-1607) cellular proliferation. Nitric oxide is normally produced by the vascular endothelium and, because of a very short half-life ($t_{1/2}$ in seconds), diffuses only to the adjacent smooth muscle where it causes relaxation via the activation of soluble guanylate
10 cyclase (Moncada, et al., 1991, *Pharmacol. Rev.* 43: 109-142). Nitric oxide released toward the lumen assists in preventing platelet adherence. L-arginine serves as the substrate for nitric oxide formation, and the small amounts of nitric oxide derived from endothelial cells is produced in an ongoing fashion (Palmer, et al., 1987, *Nature* 327: 524-526; Ignarro, et al., 1987, *Proc. Natl. Acad. Sci. USA* 84: 9265-9269) by a cNOS,
15 which is located primarily on microsomal and plasma membranes. Agonists such as acetylcholine and bradykinin increase cNOS by activity enhancing calcium/calmodulin binding to the enzyme. The cDNA coding for this enzyme has been cloned from human endothelial cells. (Janssens, et al., 1992, *J. Biol. Chem.* 267: 14519-14522; Marsden, et al., 1992, *FEBS Letters* 307: 287-293).

20 We recently demonstrated that nitric oxide biosynthesis is induced in isolated human hepatocytes after stimulation with interleukin-1, tumor necrosis factor-alpha, interferon-gamma and bacterial lipopolysaccharide (bacterial endotoxin) [Nussler, et al., April 1992, *FASEB J.* 6(5): A1834 and Nussler, et al., 1992, *J. Exp. Med.* 176: 261-264)]. Heretofore no human cell type was known to show increased production of
25 nitrogen oxides typical of iNOS expression when treated with cytokines (Drapier, 1991, *Res. Immunol.*, Vol. 142: 557). It is generally known by those skilled in the art that all attempts to induce nitric oxide synthase in human macrophages and related cells typical to those found in rodent macrophages have failed (Drapier, *Res. Immunol.* 142: 562, 589-590). In spite of this background material, there remains a very real and substantial
30 need for a cDNA clone for human tissue inducible nitric oxide synthase and a process for the molecular cloning of the same.

Inducible NOS can be expressed in cell types such as murine macrophages under conditions of cytokine and endotoxin activation (Stuehr, et al., 1985, Proc. Natl. Acad. Sci. USA 82: 7738-7742; Hibbs, et al., 1987, Science 235: 473-476; Hibbs, et al., 1987, J. Immunol. 248: 550-565), rat hepatocytes (Curran, et al., 1989, J. Exp. Med. 170: 1769-1774; Billiar, et al., 1990, Biophys. Res. Com. 168: 1034-1040), rat vascular smooth muscle cells (Busse, et al., 1990, FEBS Letter 275: 87-90; Nakayama, et al., 1992, Am. J. Respir. Cell. Mol. Biol. 7: 471-476), and capillary endothelial cells (Gross, et al., 1991, Biochem. Biophys. Res. Com. 179: 823-829). This enzyme, which is completely absent in resting cells, produces large amounts of nitric oxide continuously over many hours. A calmodulin is tightly bound to the iNOS molecule, keeping the enzyme in a fully activated state (Cho, et al., 1992, J. Exp. Med. 176: 599-604; Iida, et al., 1992, J. Biol. Chem. 267: 25385-25388). Release of large amounts of nitric oxide after iNOS induction in macrophages has cytostatic properties and is involved in the prevention of tumor cell (Hibbs, et al., 1987, Science 235: 473-476) and microbial (Green, et al., 1992, J. Leuk. Biol. 50: 93-103) proliferation. Despite iNOS related systemic toxicity seen in various tissues, it would be advantageous to target local cell populations with a DNA sequence encoding iNOS or a biologically active fragment or derivative; such a gene therapy treatment will promote prophylactic and/or therapeutic actions in regard to diseases or disorders including but not necessarily limited to vascular occlusive disease, tumor cell growth associated with cancer, and numerous microbial infections.

3. SUMMARY OF THE INVENTION

The present invention has met the hereinbefore described needs. The present invention provides a cDNA clone for human tissue inducible nitric oxide synthase and a process for preparing the same.

5 More specifically, this invention provides a cDNA clone for human hepatocyte inducible nitric oxide synthase and a process for preparing the same. This process includes inducing nitric oxide synthase expression in human hepatocytes, identifying the presence of human hepatocyte nitric oxide synthase messenger RNA, collecting the human hepatocyte poly A messenger RNA, constructing a cDNA library from the human
10 hepatocyte poly A messenger RNA, screening this cDNA library for human hepatocyte inducible nitric oxide synthase cDNA clones, and converting the human hepatocyte inducible nitric oxide synthase cDNA clones to a plasmid vector for obtaining a full length cDNA clone encoding human hepatocyte inducible nitric oxide synthase. This process further includes sequencing this cDNA, expressing the human hepatocyte
15 inducible nitric oxide synthase cDNA as a protein in an expression system, and purifying the human hepatocyte inducible nitric oxide synthase protein encoded by the cDNA.

In a further embodiment of the invention, a cDNA clone encoding inducible nitric oxide synthase (iNOS) or a biologically active fragment or derivative thereof will be utilized in gene therapy techniques to treat any number of maladies effected by nitric
20 oxide, including but not solely limited to (1) vascular occlusive disease associated with atherosclerosis; (2) resist vascular conduit occlusion due to thrombosis, intimal hyperplasia, or atherosclerosis; (3) treatment of accelerated vascular occlusive disease associated with diabetes mellitus which results in a high incidence of myocardial infarction, renal failure, stroke, blindness and limb loss at an early age; (4) treatment of
25 cancer, specifically as an antitumor agent by increasing local nitric oxide concentrations in and around the tumor(s); and (5) treatment of various microbial infections.

In regard to treatments (1), (2) and (3) disclosed in the previous paragraph (herein referred to as vascular diseases or vascular disorders), local tissue specific expression of iNOS in targeted cells will result in the production of effective amounts of nitric oxide in
30 the area of expression, so as to promote maximal local vasodilation, resist local thrombosis and potentially retard local smooth muscle cell proliferation, all of which may prevent the atherosclerotic disease process. It will be understood to one of ordinary skill

in the art that any nucleic acid sequence encoding an inducible form of NOS, preferably human iNOS, regardless of the tissue source, is a candidate for utilization in, for example, gene therapy of vascular occlusive complications associated with atherosclerosis, vascular bypass, and diabetes derived vascular disease at sites of anastomosis. It will be further understood by the skilled artisan that any nucleic acid sequence which encodes a biologically active form of iNOS, preferably a human form of iNOS, including but not limited to a genomic or cDNA sequence or a fragment thereof which encodes a biologically active protein fragment or derivative, may be utilized in the present invention.

The present invention discloses treatment of vascular diseases or vascular disorders by increasing local iNOS activity, and thus nitric oxide concentrations, through targeting of mammalian cell populations which comprise the luminal lining of the arterial vessel, namely endothelial cells and vascular smooth muscle cells. More specifically, the target mammalian cells may be, but are not necessarily limited to: (1) *in vitro* cultured endothelial cells and (2) *in vitro* cultured vascular smooth muscle cells. These cells may be transduced with a DNA sequence encoding iNOS or a biologically active fragment or derivative thereof and may be subsequently utilized to repopulate arterial vessels of the patient. It is also within the scope of this invention to use iNOS-expressing endothelial cells, vascular smooth muscle cells or a combination of both to repopulate a diseased vessel or to seed a synthetic or autologous graft.

It will be preferred to utilize endothelial and/or smooth muscle cells obtained from the patient, which may be isolated and cultured by any number of methods known to one of ordinary skill in the art. A direct source of these arterial lumen cells may be obtained, for example, by harvesting a portion of a saphaneous vein or any other accessible vein or artery from the patient. This mode of obtaining target cell source material for *in vitro* culture prior to iNOS infection or transfection procedures will be especially useful in seeding a synthetic or autologous graft for transfer to the patient.

In another embodiment of the present invention, endothelial cells, vascular smooth muscle cells or a combination of both are targeted for *in situ* infection or transfection with a DNA sequence encoding iNOS or a biologically active fragment or derivative thereof so as to promote increased local iNOS expression within selected segments of arterial vessels.

It will be understood by the skilled artisan that similar procedures may be utilized for *in vitro* transfection or infection of endothelial cells and vascular smooth muscle cells. Both endothelial cells and vascular smooth muscle cells may be infected simultaneously through an *in situ* procedure, exemplified but not limited to the procedure outlined in
5 Example Section 13.1.2.

It will also be understood that one or more endovascular procedures available to the skilled vascular surgeon may be utilized to prepare the diseased vessel for iNOS-based gene therapy as well as to deliver the DNA sequence encoding iNOS to the conduit area targeted for treatment. Such procedures, alone or in combination, include but are not
10 necessarily limited to balloon angioplasty, laser-assisted balloon angioplasty, double balloon catheterization, mechanical endarterectomy and vascular endoscopy.

It will also be understood by the skilled artisan that a combination of strategies disclosed further within this specification may be utilized in conjunction with surgical vascular bypass procedures to promote a gene therapy based increase in local iNOS
15 expression at sites of surgical repair or within a synthetic graft.

In a particular embodiment regarding targeting of *in vitro* cultured endothelial cells, vascular smooth muscle cells or a combination of both for gene therapy of vascular diseases, a DNA sequence encoding iNOS or a biologically active fragment thereof will be ligated to a viral vector in preparation for tissue specific delivery and expression.

20 Virus vector systems utilized in the present invention include, but are not limited to (a) retroviral vectors, including but not limited to vectors derived from the Moloney murine leukemia virus (MoMLV) genome; (b) adeno-associated vectors; (c) adenovirus vectors; (d) herpes simplex virus vectors; (e) SV40 vectors; (f) polyoma virus vectors; (g) papilloma virus vectors; (h) picornavirus vectors; and (i) vaccinia virus vectors.

25 Additional strategies which the skilled artisan may utilize alone or in combination with viral vectors in targeting endothelial cells, vascular smooth muscle cells or a combination thereof for gene therapy of vascular diseases include but are not limited to (a) liposome-mediated transformation; (b) calcium phosphate $[Ca_3(PO_4)_2]$ mediated cell transfection; (c) *in vitro* transfection of target cells by electroporation; (d) DEAE-dextran
30 mediated cell transfection, the *in vitro* transfected cells then utilized to repopulate the mammalian host; (e) polybrene mediated delivery; (f) protoplast fusion; (g) microinjection; (h) polylysine mediated transformation; and (i) direct injection of

naked DNA. The genetically transformed cells generated by any of these strategies are then utilized to repopulate the mammalian host.

In a particular embodiment regarding the treatment of vascular diseases, a recombinant viral vector comprising a DNA sequence encoding iNOS or a biologically active fragment or derivative utilized to infect mammalian endothelial cells, vascular smooth muscle cells or a combination of both for repopulation of arterial vessels is a recombinant retroviral vector. The respective iNOS DNA sequence is ligated within the retroviral vector to form a retroviral-iNOS recombinant construct.

In a preferred embodiment regarding the treatment of vascular diseases, the iNOS sequence subcloned into an appropriate retroviral vector is a human iNOS sequence.

In a further preferred embodiment regarding use of a retroviral vector in gene therapy of vascular diseases, the recombinant retroviral vector is a MoMLV-iNOS construct. This iNOS containing retroviral construct comprises a human DNA sequence encoding iNOS or a biologically active fragment or derivative thereof.

In a preferred embodiment regarding use of a retroviral vector in gene therapy of vascular diseases, the MoMLV-iNOS construct is MFG-iNOS as depicted in Figure 6 and Figure 7. The MFG-iNOS construct is preferred for *in situ* infection of the target cell.

In another preferred embodiment regarding use of a retroviral vector in gene therapy of vascular diseases, the MoMLV-iNOS construction is DFG-iNOS-Neo as depicted in Figure 6 and Figure 8. The DFG-iNOS-Neo construct is preferred for *in vitro* infection of endothelial cells or vascular smooth muscle cells.

Any of the hereinbefore disclosed retroviral-iNOS recombinant constructs are then transferred into a standard retroviral packaging cell line. The recovered recombinant viral particles are then used to infect cultured endothelial cells or vascular smooth muscle cells *in vitro*. Treatment of vascular diseases is based further on transferring *in vitro* transduced or infected endothelial cells, vascular smooth muscle cells or a combination of both to specific segments of diseased arteries within the patient. A preferred mode of delivering infected endothelial cells, vascular smooth muscle cells or a combination of both utilizes a double balloon catheter to isolate a segment of a mammalian host artery which has been partially or totally denuded of its endothelial cell lining as often encountered following a balloon angioplasty procedure, endarterectomy or following surgical vascular bypass.

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In vitro viral-mediated infection or vector-mediated transfection of endothelial cells or vascular smooth muscle cells with a DNA sequence encoding iNOS or a biologically active fragment thereof may be accomplished by numerous non-biologic and/or biologic carriers other than the hereinbefore mentioned retroviral vectors. Therefore, any non-biologic and/or biologic carrier possessing the ability to deliver an iNOS encoding DNA sequence to the local target such that iNOS is expressed at therapeutic or prophylactic levels may be utilized to practice the present invention.

For example, in an additional embodiment of the invention, a DNA sequence encoding iNOS or a biologically active fragment thereof may be subcloned into an adenoviral vector (Ad). The recombinant Ad-iNOS construct can be utilized to directly infect *in vitro* cultured endothelial cells, vascular smooth muscle cells or a combination thereof, or alternatively, can be delivered to the target cells through the association with liposome microcapsules.

Another embodiment of the invention involves a DNA sequence encoding iNOS or a biologically active fragment thereof which may be subcloned into an adeno-associated viral vector (AAV). As with an Ad-iNOS construct, the recombinant AAV-iNOS construct can be utilized to directly infect *in vitro* cultured endothelial cells, vascular smooth muscle cells or a combination thereof, or alternatively, can be delivered to the target cells through the association with liposome microcapsules.

In a further embodiment regarding the use of liposome-mediated techniques to deliver recombinant iNOS constructs to treat vascular diseases, a viral or non-viral vector comprising a DNA sequence encoding iNOS is delivered to the target cell by lipofectamine transfection. For example, a DNA sequence encoding iNOS or a biologically active fragment thereof is subcloned into a DNA plasmid vector such that iNOS is expressed subsequent to transfection of the target cell. Such non-viral based mammalian vectors include, but are not solely limited to, a plasmid DNA mammalian expression vector. Any eukaryotic promoter and/or enhancer sequence available to the skilled artisan which is known to up-regulate expression of iNOS may be used in mammalian expression vector constructs, including but not limited to a cytomegalovirus (CMV) promoter, a Rous Sarcoma (RSV) promoter, a Murine Leukemia (MLV) promoter, a herpes simplex virus (HSV) promoter, such as HSV-tk, a β -actin promoter, as well as any additional tissue specific or signal specific regulatory sequence that induces

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expression in the target cell or tissue of interest. A signal specific promoter fragment includes but is not limited to a promoter fragment responsive to TNF.

In one such embodiment, a DNA sequence encoding human iNOS is subcloned into the DNA plasmid expression vector, pCIS (Genentech), resulting in pCIS-iNOS.

5 pCIS is a standard mammalian expression vector, containing an antibiotic resistance gene for propagation in *E. coli* and a CMV promoter active in mammalian cells. Such a construct, which may be constructed by one of ordinary skill with components available from numerous sources, will drive expression of an iNOS DNA fragment ligated downstream of the CMV promoter subsequent to transfection of the target cell. More
10 specifically, a NotI/XhoI restriction fragment containing the human iNOS coding region is generated and isolated from pHINOS (pHINOS is deposited with the ATCC with accession number 75358) and ligated into NotI/XhoI digested pCIS. Alternatively, the isolated human iNOS sequence may be fused to any portion of the wild type human iNOS promoter sequence such that expression of human iNOS can be induced within the target
15 cell.

It will become evident to one of ordinary skill in the art upon review of this specification that any of the viral or non-viral recombinant iNOS constructs hereinbefore described for use in infecting or transfecting *in vitro* cultured endothelial cells, vascular
20 smooth muscle cells or a combination thereof may be used to infect or transfect target cell *in situ*. For example, balloon angioplasty may be utilized to dilate an occluded segment of diseased arterial vessel so as to reestablish the arterial lumen. The dilated segment is then segregated from the remainder of the arterial vessel by inserting a double balloon catheter. A viral or non-viral based recombinant iNOS construct may be selectively delivered through the catheter to the angioplasty site so as to promote *in situ* transfection
25 or infection of endothelial and/or vascular smooth muscle cells with concomitant local increases in iNOS expression within the diseased vessel segment.

The present invention also discloses methods of human iNOS-directed gene therapy to promote antitumor effects in cancer patients. Such a human iNOS-directed gene therapy will provide a local increase in nitric oxide concentration within the area of
30 the tumor to be treated, thus promoting antitumor activity without systemic increases in nitric oxide levels. As disclosed for iNOS-mediated treatment, a human derived DNA

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sequence encoding iNOS or a biologically active fragment or derivative thereof is preferred.

The isolated human iNOS DNA sequence may be manipulated and delivered to the target cell *in vitro* by transduction utilizing any of the viral and non-viral methods discussed in Section 5.2.1. The *in vitro* transduced target cells are then introduced into the patient so as to promote local iNOS expression at the tumor site. Therefore, it will be understood that any human iNOS DNA sequence encoding a biologically active fragment or derivative thereof, regardless of tissue source, is a candidate for antitumor treatments.

In one embodiment regarding cancer gene therapy, the patient is intravenously injected with *in vitro* transduced target cells, including but not limited to tumor infiltrating lymphocytes or cultured tumor cells harvested from the patient.

In a preferred method of delivering a human iNOS sequence to the target cell of interest, a recombinant retroviral vector comprising a DNA sequence encoding iNOS or a biologically active fragment thereof is utilized to infect tumor infiltrating lymphocytes. These infected tumor infiltrating lymphocytes are then reintroduced into the patient to promote local expression of iNOS at the tumor site.

In a preferred embodiment regarding gene therapy of cancer, DFG-iNOS-Neo (Figure 8) is utilized to infect tumor infiltrating lymphocytes or cultured tumor cells harvested from the patient. Neomycin resistant cells are selected, followed by localization of these iNOS expressing cells to the region within and surrounding the active tumor.

In addition to the hereinbefore described use of viral vectors to infect target cells, any known non-viral vector described in this specification may be utilized to promote antitumor activity.

The human iNOS DNA sequences of the present invention may also be utilized in treating microbial infections. Specifically, iNOS-driven antimicrobial therapy will be utilized to treat microbes known to be susceptible to increased concentrations of nitric oxide. For example, nitric oxide is known to be a cytotoxic effector molecule against mycobacteria, helminths, fungi, protozoa and DNA viruses. Therefore, the present invention discloses methods of increasing concentrations of nitric oxide locally at the site of infection by targeting the infected cell or tissue type with a DNA sequence encoding

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iNOS activity, preferably human iNOS, capable of being expressed at a therapeutic level and duration so as to surmount the disease.

In a preferred embodiment of utilizing iNOS-driven antimicrobial therapy, the target cell type is human hepatocytes infected with the sporozoa *Plasmodium*, the causative agent of malaria. Human malaria is caused by one of four species of *Plasmodium*: *P. falciparum*, *P. malariae*, *P. vivax* and *P. ovale*.

In a preferred embodiment of treating malaria via iNOS-antimicrobial therapy, the iNOS-vector is delivered via liposome mediated transformation of the target hepatocytes.

In an especially preferred embodiment of treating malaria via iNOS-antimicrobial therapy, the liposomes are modified by insertion of an hepatocyte specific asialoprotein into the liposome membrane prior to administration to the patient.

Another embodiment of utilizing iNOS-vectors in antimicrobial therapy involves treatment of lung borne microbial infections, including but not limited to tuberculosis and leprosy.

A preferred treatment of tuberculosis by iNOS-antimicrobial therapy involves targeting an iNOS vector to the target tissue by viral mediated transformation of cells within the target tissue.

A preferred method of treating tuberculosis by iNOS-antimicrobial therapy is adenovirus-mediated delivery to the site of infection.

Another preferred method of treating tuberculosis by iNOS-driven biologic therapy is retroviral mediated delivery, as discussed in Section 5.2.1. iNOS-based vectors disclosed in Sections 5.2.1 and 5.2.2 may also be utilized in retroviral-mediated delivery techniques to treat tuberculosis.

With the aid of this specification, it would be within the realm of the artisan of ordinary skill to construct an iNOS vector compatible with the delivery system of choice for use in treating tuberculosis.

A preferred method of administering an iNOS-infected retrovirus within infected regions of lung tissue is inhalatory administration, in the form of an aerosol mist.

Another embodiment of the invention relates to treatment of *Mycobacterium leprae*, the causative agent of leprosy. The preferred mode of treating leprosy by gene therapy entails retroviral-mediated transduction of target tissue cell types by inhalatory administration.

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It is an object of the present invention to provide for the molecular cloning and characterization of an inducible nitric oxide synthase in human tissues.

It is an object of the present to provide for the molecular cloning and characterization of an inducible nitric oxide synthase from human hepatocytes.

5 It is an object of the present invention to isolate a cDNA clone for human tissue inducible nitric oxide synthase.

It is an object of the present invention to isolate a cDNA clone for human hepatocyte inducible nitric oxide synthase.

10 It is an object of the present invention to provide a process for expressing and purifying human tissue inducible nitric oxide synthase enzyme.

It is an object of the present invention to provide a process for expressing and purifying human hepatocyte inducible nitric oxide synthase enzyme.

It is an object of this invention to provide for the regulation of gene expression for the human hepatocyte inducible nitric oxide synthase enzyme.

15 It is an object of this invention to provide for a protein including a human inducible nitric oxide synthase substantially free of other human proteins.

It is an object of this invention to promote vascular gene therapy to provide prophylactic and therapeutic relief from vascular diseases including but not limited to vascular occlusive diseases associated with atherosclerosis, vascular bypass, and
20 associated with diabetes by providing transfected endothelial cells, vascular smooth muscle cells or a combination of both which express iNOS or a biologically active fragment thereof to a patient's diseased blood vessel, a vascular conduit, or blood vessel partly or totally denuded of its endothelial lining.

It is an object of this invention to provide therapeutic treatment of tumor growth
25 by utilizing iNOS-driven gene therapy techniques to increase local nitric oxide concentrations so as to inhibit tumor growth.

It is an object of this invention to provide therapeutic relief from various microbial infections susceptible to attack by utilizing iNOS-driven gene therapy techniques to
30 increase local concentration of nitric oxide at or around the site of infection, especially the various pulmonary and hepatic infections described in this specification.

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These and other objects of the invention will be more fully understood from the following description of the invention, the figures, the sequence listing and the claims appended hereto.

3.1. DEFINITIONS

The terms listed below, as used herein, will have the meaning indicated.

mRNA -	messenger RNA
DNA -	deoxyribonucleic acid
cDNA -	complementary deoxyribonucleic acid
NO -	nitric oxide
NOS -	nitric oxide synthase
cNOS -	constitutive nitric oxide synthase
iNOS -	inducible nitric oxide synthase
EDRF -	endothelium derived relaxing factor
LPS -	lipopolysaccharide
CMV -	cytomegalovirus
Ad -	adenovirus
AAV -	adeno-associated virus
IRES -	internal ribosome entry site
PTFE -	polytetrafluoroethylene

As used herein, the term "patient" includes members of the animal kingdom including but not limited to human beings.

As used herein, the term "mammalian host" includes mammals, including but not limited to human beings.

As used herein, the term "biologically active fragment or derivative thereof" includes any iNOS protein fragment possessing similar biological activity as wild type iNOS, or a derivative such as an iNOS substitution, addition and/or deletion mutant which maintains similar biological activity as wild type iNOS. One of ordinary skill in the art may use the present specification to generate such changes in the wild type iNOS DNA sequence so as to express variants of wild type iNOS which retain the biological activity necessary to be useful in the presently disclosed gene therapy applications.

4. BRIEF DESCRIPTION OF THE FIGURES

Figures 1A-G show the cDNA sense sequence (top line of each horizontal row; SEQ ID NO:1) and the amino acid sequence of amino acids 1-1153 (bottom line of each horizontal row; SEQ ID NOS: 1 and 2) for the cDNA clone for human hepatocyte inducible nitric oxide synthase.

Figure 2 shows a Northern blot of a mouse macrophage NOS cDNA cross-hybridizing to human hepatocyte (HC) nitric oxide synthase mRNA.

Figure 3 shows a Northern blot of induced nitric oxide synthase mRNA isolated from three separate human liver samples using mouse macrophage cDNA.

Figure 4 shows a Northern blot of poly A mRNA purified from 2 separate human liver samples used in the construction of the cDNA library for isolation of the cDNA clone for the human hepatocyte inducible nitric oxide synthase.

Figure 5 shows a Northern blot using cDNA isolated from human hepatocytes that sets forth the time course of induction of human nitric oxide synthase mRNA following cytokine and LPS stimulation.

Figure 6 shows the MFG-iNOS and DFG-iNOS-Neo recombinant retroviral vectors utilized to exemplify the gene therapy applications to treat diseases or disorders disclosed throughout this specification. Neo^r encodes resistance to neomycin; the IRES fragment allows translation of a polycistronic mRNA; LTR are long terminal repeats of the MoMLV genome; iNOS is the cDNA encoding human hepatocyte iNOS.

Figure 7 shows detailed methods utilized to construct MFG-iNOS, a recombinant retroviral vector utilized to exemplify various gene therapy applications disclosed throughout this specification.

Figure 8 shows detailed methods utilized to construct DFG-iNOS-Neo, a recombinant retroviral vector utilized to exemplify various gene therapy applications disclosed throughout this specification.

Figure 9 shows nitrite production in cultured endothelial cells infected with MFG-iNOS, MFG-lacZ and uninfected cells in the absence and presence of the iNOS inhibitor, N^G-monomethylarginine.

Figure 10 shows nitrite production in cultured endothelial cells infected with DFG-iNOS-Neo, MFG-lacZ and uninfected cells in the absence and presence of the iNOS inhibitor, N^G-monomethylarginine.

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Figure 11 shows nitrite production in vascular smooth muscle cells after lipofectamine transfection of pCIS-iNOS in the absence and presence of N^G-monomethylarginine, pSV-lacZ, and a plasmid-less control with or without the addition of liposomes.

5. DETAILED DESCRIPTION OF THE INVENTION

Nitric oxide is a biologic mediator derived from the amino acid L-arginine. Nitric oxide synthase (NOS) acts upon L-arginine to oxidize one of the guanidino nitrogens to nitric oxide while citrulline is formed from the remainder of the L-arginine molecule.

5 While it is understood by those skilled in the art that nitric oxide has normal physiologic intracellular and extracellular regulatory functions, excessive production of nitric oxide can be both detrimental and beneficial. It will be appreciated by those skilled in the art that there are no other readily available sources of human tissue inducible nitric oxide synthase. The present invention provides a cDNA clone for human tissue inducible nitric
10 oxide synthase and a process for preparing the same. Therefore, the cloning and expression of a human tissue nitric oxide synthase cDNA of the present invention provides for a source of the enzyme for developing selective inhibitors of nitric oxide synthase. The present invention also relates to gene therapy techniques utilizing a human iNOS DNA sequence to provide therapeutic relief from diseases or disorders such as
15 vascular occlusive disease associated with atherosclerosis, vascular bypass, and diabetes mellitus, tumor cell growth associated with cancer, and microbial infections.

The cloning and expression of a human tissue nitric oxide synthase cDNA of the present invention provides for a source of the enzyme in a sufficiently high concentration for providing a therapeutic purpose.

5.1. ISOLATION AND CHARACTERIZATION OF A cDNA CLONE CODING FOR A HUMAN INDUCIBLE NITRIC OXIDE SYNTHASE

In one embodiment of this invention, a process for preparing a cDNA clone coding for a human tissue inducible nitric oxide synthase is provided. This process
25 includes inducing the expression of human tissue nitric oxide synthase *in vitro*, identifying the presence of human tissue nitric oxide synthase messenger RNA (mRNA) by employing a cross-species cDNA probe capable of hybridizing with the human tissue inducible nitric oxide synthase mRNA, collecting the human tissue poly A mRNA which included the human tissue nitric oxide synthase mRNA, constructing a cDNA library from
30 the human tissue poly A mRNA using a reverse transcriptase enzyme and inserting a strand of the cDNA into a phage vector, screening the cDNA library for human tissue inducible nitric oxide synthase clones with a cross-species iNOS cDNA probe, incubating

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the phage vector containing the cDNA with a bacteria for forming at least one positive plaque containing the cDNA clone for human tissue inducible nitric oxide synthase, rescuing the cDNA clone from the phage vector by employing a helper phage, and converting the rescued cDNA clone to a plasmid vector for obtaining a full length cDNA clone encoding human tissue inducible nitric oxide synthase.

In another embodiment of this invention, this process, as hereinbefore described, further includes excising the cDNA insert for human tissue inducible nitric oxide synthase from the plasmid vector. This process also includes confirming the cDNA insert by employing dideoxynucleotide DNA sequencing. Further, this process includes confirming the cDNA insert by employing Southern blot hybridization with a cross-species cDNA probe derived from murine macrophage iNOS.

In another embodiment of this invention, the process, as hereinbefore described, includes expressing the human tissue inducible nitric oxide synthase cDNA protein in an expression system, such as for example, a bacterial expression system or a mammalian expression system.

It will be appreciated by those skilled in the art that the cloned human inducible nitric oxide synthase cDNA obtained through the methods described herein may be recombinantly expressed by molecular cloning into an expression vector containing a suitable promoter and other appropriate transcription regulatory elements, and transferred into prokaryotic or eukaryotic host cells to produce recombinant inducible nitric oxide synthase. Techniques for such manipulations are fully described in Maniatis, et al., *infra*, and are well known in the art.

Expression vectors are defined herein as DNA sequences that are required for the transcription of cloned copies of genes and the translation of their mRNAs in an appropriate host. Such vectors can be used to express eukaryotic genes in a variety of hosts such as for example bacteria, bluegreen algae, plant cells, insect cells and animal cells.

Specifically designed vectors allow the shuttling of DNA between hosts such as bacteria-yeast or bacteria-animal cells. An appropriately constructed expression vector should contain: an origin of replication for autonomous replication in host cells, selectable markers, a limited number of useful restriction enzyme sites, a potential for high copy number, and active promoters. A promoter is defined as a DNA sequence that directs

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RNA polymerase to bind to DNA and initiate mRNA synthesis. A strong promoter is one which causes mRNAs to be initiated at high frequency. Expression vectors may include, but are not limited to, cloning vectors, modified cloning vectors, specifically designed plasmids or viruses. A variety of mammalian expression vectors may be used to express recombinant inducible nitric oxide synthase in mammalian cells.

Commercially available bacterial expression vectors which may be suitable for recombinant inducible nitric oxide synthase expression, include but are not limited to, pKC30 (ATCC 37286), pPLa2311 (ATCC 31694), pBR322 (ATCC 31344 and 37017), ptac12 (ATCC 37138), lambda gt11 (ATCC 37194), pAS1 (ATCC39262), pLC24, pSB226, SV40 and pKK 223-3.

Commercially available mammalian expression vectors which may be suitable for recombinant inducible nitric oxide synthase expression, include but are not limited to, pBC12B1 (ATCC 67617), pMCIneo (Stratagene), pXTI (Stratagene), pSG5 (Stratagene), EBO-pSV2-neo (ATCC 37593) pBPV-1(8-2) (ATCC 37110), pdBPV-MMTneo(342-12) (ATCC 37224), pRSVgpt (ATCC 37199), pRSVneo (ATCC 37198), pSV2-dhfr (ATCC 37146), pUCTag (ATCC 37460), and lambda ZD35 (ATCC 37565).

DNA encoding inducible nitric oxide synthase may also be cloned into an expression vector for expression in a recombinant host cell. Recombinant host cells may be prokaryotic or eukaryotic, including but not limited to bacteria, yeast, mammalian cells including but not limited to cell lines of human, bovine, porcine, monkey and rodent origin, and insect cells including but not limited to drosophila derived cell lines. Cell lines derived from mammalian species which may be suitable and which are commercially available, include but are not limited to, CV-1 (ATCC CCL70), COS-1 (ATCC CRL1650), COS-7 (ATCC CRL1651), CHO-K1 (ATCC CCL61), 3T3 (ATCC CCL92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL2), C1271 (ATCC CRL1616), BS-C-1 (ATCC CCL26) and MRC-5 (ATCC CCL171). The bacterial cell most used for expression of recombinant protein is *Escherichia coli*. There are various strains of *E. coli* available and are well known in the art.

The expression vector may be introduced into host cells via any one of a number of techniques including but not limited to transformation, transfection, infection, protoplast fusion, and electroporation.

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In a preferred embodiment of this invention, the process, as hereinbefore described, includes expressing the human tissue inducible nitric oxide synthase protein in a baculovirus expression system.

Another embodiment of this invention provides for a process, as hereinbefore described, including purifying the human tissue inducible nitric oxide synthase protein.

In a preferred embodiment of this invention, the process, as hereinbefore described, includes employing as the human tissue inducible nitric oxide synthase a human hepatocyte inducible nitric oxide synthase. This process further includes employing as the human tissue inducible nitric oxide synthase protein a human hepatocyte inducible nitric oxide synthase protein.

In another embodiment of this invention, a process is provided, as hereinbefore described, including inducing the human tissue nitric oxide synthase *in vitro* by stimulating a human tissue *in vitro* with at least one of the following (1) at least one cytokine, such as for example a cytokine selected from the group consisting of tissue necrosis factor (TNF), interleukin-1 (IL-1), and interferon-gamma (IFN-g), (2) at least one bacterial endotoxin including, such as for example, a bacterial lipopolysaccharide (LPS) and (3) combinations thereof.

A further preferred embodiment of this invention provides a process, as hereinbefore described, that includes constructing a cDNA library from the human tissue poly A mRNA which includes the human tissue iNOS mRNA using a reverse transcriptase enzyme and inserting cDNA strands having a length of about at least 1,000 base pairs into the phage vector. In yet another preferred embodiment, a process is provided, as hereinbefore described, that includes employing lambda Zap II as the phage vector.

In another embodiment of this invention, a process is provided, as hereinbefore described, including screening the cDNA library by incubating the phage vector for about 6 to 24 hours with a bacteria at a temperature of about 34 to 40 degrees centigrade for effectuating phage lysis of the bacteria. This process further includes rescuing the cDNA clone from the phage vector by employing a helper phage such as for example ExAssist helper phage (Stratagene, La Jolla, CA).

In a preferred embodiment of this invention, a process, as hereinbefore described, is provided including converting the rescued cDNA clone to the plasmid vector for

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obtaining a full length cDNA clone encoding the human tissue inducible nitric oxide synthase wherein the plasmid vector includes pBluescript (Stratagene, La Jolla, CA).

In another preferred embodiment of this invention, a process, as hereinbefore described, is provided including employing as the human tissue inducible nitric oxide synthase a human hepatocyte inducible nitric oxide synthase.

Another embodiment of this invention provides for a process for producing human hepatocyte inducible nitric oxide synthase protein comprising providing a replicatable DNA expression vector capable of expressing a DNA sequence encoding human hepatocyte inducible nitric oxide synthase in a suitable host, transforming the host to obtain a recombinant host, and maintaining the recombinant host under conditions permitting expression of the DNA sequence to provide human hepatocyte inducible nitric oxide synthase protein.

Another embodiment of this invention provides a human tissue inducible nitric oxide synthase cDNA clone. A preferred embodiment of this invention provides a human hepatocyte inducible nitric oxide synthase cDNA clone. The human hepatocyte inducible nitric oxide synthase cDNA clone of this invention has a cDNA coding for the amino acid sequence shown in Figures 1A-G. Figures 1A-G show the cDNA sense sequence (top line of each horizontal row; SEQ ID NO:1) and the deduced amino acid sequence of amino acids 1-1153 (bottom line of each horizontal row: SEQ ID NOS:1 and 2) for the cDNA clone for the human hepatocyte inducible nitric oxide synthase of this invention. Figures 1A-G show that the cDNA sequence for the human hepatocyte inducible nitric oxide synthase of this invention is 4,145 nucleotide bases long with the start codon beginning at base number 207 and the stop codon ending at base number 3668. The cDNA double strand sequence was determined using the Sanger dideoxynucleotide sequence technique (Sanger, et al., 1977, Proc. Natl. Acad. Sci. USA 74: 5463-5467) on a Genesis 2000 sequencing system (USB, Cleveland, Ohio).

Another embodiment of this invention provides a human tissue inducible nitric oxide synthase recombinant protein expressed from a human tissue inducible nitric oxide synthase cDNA clone. In a preferred embodiment, a human hepatocyte inducible nitric oxide synthase recombinant protein expressed from a human hepatocyte inducible nitric oxide synthase cDNA clone is provided.

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Another embodiment of this invention provides for a protein comprising a human inducible nitric oxide synthase substantially free of other human proteins.

Another embodiment of this invention provides for an isolated DNA sequence encoding human inducible nitric oxide synthase consisting essentially of an initiation codon positioned upstream and adjacent to an open reading frame consisting essentially of a DNA sequence encoding human inducible nitric oxide synthase.

A further embodiment of this invention provides for an isolated DNA sequence encoding human inducible nitric oxide synthase consisting essentially of an initiation codon positioned upstream and adjacent to an open reading frame consisting essentially of a DNA sequence encoding human inducible nitric oxide synthase protein. The human inducible nitric oxide synthase protein begins at the initiation codon and terminates at a stop codon.

In yet another embodiment of this invention, a recombinant plasmid is provided containing a recombinant plasmid pHINOS having a deposit accession number ATCC 75358 deposited with the American Type Culture Collection. A further embodiment of this invention provides for bacteria transformed by the recombinant plasmid pHINOS.

In another embodiment of this invention a microorganism is provided containing a HINOS cDNA plasmid transformed in *E. coli* SOLR bacteria having a deposit accession number ATCC 69126 deposited with the American Type Culture Collection.

5.2. GENE THERAPY APPLICATIONS UTILIZING HUMAN iNOS

5.2.1. VASCULAR GENE THERAPY UTILIZING HUMAN iNOS

Vascular occlusive disease due to atherosclerosis results in significant morbidity in the form of stroke, myocardial infarction, and limb loss. No effective means to resist these changes currently exist. The capacity to bypass occluded vessels is often limited by thrombosis and occlusion of the bypass graft.

Accelerated vascular occlusive disease associated with diabetes mellitus results in a high incidence of myocardial infarction, renal failure, stroke, blindness, and limb loss at an early age. Because smaller sized arteries are often preferentially involved, therapies such as bypass or angioplasty, aimed at alleviating stenotic vessels, are frequently ineffective or complicated by early thrombosis or early restenosis. Factors that contribute to atherosclerosis and diabetic vascular lesions include endothelial injury and dysfunction,

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macrophage and platelet accumulation, lipid and lipoprotein accumulation, accumulation of glycosylation products, and vascular smooth muscle cell proliferation (Colwell, 1991, Am. J. Med. 90: 6A-50S - 6A-54S).

5 The present invention discloses treatment of vascular diseases or vascular disorders by increasing local iNOS expression through targeting of the mammalian cell populations which form the arterial luminal lining, namely endothelial cells and vascular smooth muscle cells. More specifically, the target mammalian cells may be, but are not necessarily limited to: (1) *in vitro* cultured endothelial cells and (2) *in vitro* cultured vascular smooth muscle cells, which may be transfected or infected with a DNA sequence
10 encoding iNOS or a biologically active fragment or derivative thereof and subsequently utilized to repopulate arteries of the patient. It is also within the scope of this invention to use a combination of infected and/or transfected endothelial and vascular smooth muscle cells for repopulation of a diseased vessel.

15 It will be preferred to utilize endothelial and/or smooth muscle cells obtained from the patient, which will be placed into culture by any number of methods known to one of ordinary skill in the art. A source of these arterial-based cells may be obtained by harvesting a source these cells from the patient, including but not limited to harvesting a portion of a saphaneous vein from the patient. This mode of obtaining target cell source material for *in vitro* culture prior to iNOS infection or transfection procedures will be
20 especially useful in seeding a synthetic or autologous graft for transfer to the patient.

In another embodiment of the present invention, endothelial cells, vascular smooth muscle cells or a combination of both are targeted for *in situ* infection or transfection with a DNA sequence encoding iNOS or a biologically active fragment or derivative thereof so as to promote increased local iNOS expression within selected segments of arterial
25 vessels.

It is a preferred aspect of the invention to utilize a human nucleic acid fragment encoding an iNOS protein or biologically active fragment. In regard to directing a human iNOS construct to the appropriate cell type and arterial location, a further preferred embodiment involves use of the cDNA clone encoding human hepatocyte iNOS or a
30 biologically active fragment thereof. This cDNA clone may be utilized to generate various biologically active iNOS constructs for use in gene therapy applications to increase localized arterial iNOS expression for treatment of vascular diseases including

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but by no means limited to vascular occlusive disease associated with atherosclerosis and diabetes mellitus, vascular disorders resulting in a high incidence of myocardial infarction, renal failure, stroke, blindness and limb loss at an early age, as well as prevention of intimal hyperplasia. Cell and arterial specific expression of human iNOS in targeted cells will result in local production of prophylactically and therapeutically effective amounts of nitric oxide in the area of expression. Local iNOS expression will promote maximal local vasodilation, resist local thrombosis and potentially retard local vascular smooth muscle cell proliferation, all of which may resist the atherosclerotic disease process and vascular conduit occlusion. However, it will be further understood to one of ordinary skill in the art that any DNA sequence encoding an inducible form of human iNOS, regardless of the tissue source, is a candidate for utilization in gene therapy of vascular occlusive disease in humans. It will be further understood by the skilled artisan that any isolated DNA sequence encoding a protein or protein fragment which mimics the biological activity of human hepatocyte iNOS may be utilized to practice the present invention. Such isolated DNA sequences include, but are not necessarily limited to (1) an isolated cDNA or genomic fragment encoding human hepatocyte iNOS encoding a biologically active fragment thereof; (2) an isolated cDNA, genomic fragment or nucleic acid fragment encoding a biologically active protein or protein fragment of a non-hepatocyte human iNOS; (3) an isolated cDNA, genomic fragment or nucleic acid fragment thereof encoding a biologically active protein or protein fragment thereof of a non-human iNOS; or (4) a synthetic DNA molecule encoding a polypeptide fragment with similar biological activity as described for iNOS.

The DNA sequence encoding iNOS may be delivered to endothelial or vascular smooth muscle target cells by viral or non-viral mediated routes. Virus vectors utilized in the present invention include, but are not limited to (a) retroviral vectors, including but not limited to vectors derived from Moloney murine leukemia virus (MoMLV); (b) adeno-associated vectors; (c) adenovirus vectors; (d) herpes simplex virus vectors; (e) SV40 vectors; (f) polyoma virus vectors; (g) papilloma virus vectors; (h) picornavirus vectors; and (i) vaccinia virus vectors. Depending on the virus vector system chosen, techniques available to the skilled artisan are utilized to infect the target cell of choice with the recombinant viral vector.

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By way of example, and not of limitation, a recombinant retroviral vector comprising a DNA sequence encoding iNOS or a biologically active fragment thereof is utilized to infect cultured mammalian endothelial cells which are then used to repopulate arterial vessels or vascular bypass grafts. The retroviral-iNOS recombinant construct is transferred into a standard retroviral packaging cell line and the recovered viral particles are used to infect cultured endothelial cells. These *in vitro* infected cell populations are then reintroduced into the patient.

Any number of retroviral constructs which express a biologically active form of iNOS may be utilized by the skilled artisan in practicing the invention. However, a preferred embodiment of the invention depends upon infection of endothelial cells with an iNOS-containing recombinant Moloney murine leukemia virus (MoMLV) retroviral vector. Although MoMLV is a RNA virus, it has a DNA intermediate form that stably integrates into the genome of the host cell. The virus has two long terminal repeats (LTRs) at the 5' and 3' end of the proviral DNA that contain promoter, polyadenylation, and integration sequences required for the viral life cycle. A packaging sequence, termed *psi*, is also required in *cis* for the production of infectious virus. The virus encodes three proteins, *gag*, *pol*, and *env*, that are required in *trans* for viral replication. The *gag* and *pol* proteins are expressed from a non-spliced message whereas the *env* protein is expressed from a spliced message generated using the 5' and 3' splice sites shown. To generate a recombinant retroviral vector, the *gag*, *pol*, and *env* genes were removed, resulting in the replication deficient MFG derivative of MoMLV. The cDNA encoding iNOS was subcloned into MFG, resulting in MFG-iNOS. In MFG-iNOS, the gene is expressed from a LTR-driven spliced message. The MFG-iNOS construct has the *psi* site required for packaging of the recombinant RNA into virions. To generate infectious virus, the proviral DNA is transfected into a packaging cell line that constitutively produces *gag*, *pol*, and *env* proteins. Figure 1A-G shows the sequence of the cDNA encoding the human hepatocyte iNOS and is inserted into the NcoI and BamHI cloning sites of the retroviral vector MFG (Figure 6 and Figure 7; for a review of retroviral vectors, see Miller, 1992, Current Topics in Microbiology and Immunology 158: 1-24).

One of ordinary skill in the art will understand any additional isolated DNA sequence or synthetically produced DNA sequence encoding a biologically active portion of iNOS, as hereinbefore disclosed, may be subcloned into a retroviral vector for eventual

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in vitro infection of cultured endothelial cells or vascular smooth muscle cells. Infected endothelial cells or vascular smooth muscle cells may then be delivered to specific tissue target sites within the patient as described within this specification.

The present invention also discloses the use of iNOS-retroviral vectors in gene therapy applications to treat vascular disease by *in situ* infection or transfection of endothelial cells or vascular smooth muscle cells with a DNA sequence encoding iNOS so as to promote increased local iNOS expression within selected arterial segments or vascular bypass grafts.

In vitro viral-mediated infection or vector-mediated transfection of endothelial cells with a DNA sequence encoding iNOS or a biologically active fragment thereof to treat vascular disease may be accomplished by numerous non-biologic and/or biologic carriers other than the hereinbefore mentioned retroviral vectors.

For example, in an additional embodiment of the invention, a DNA sequence encoding iNOS or a biologically active fragment thereof may be subcloned into an adenovirus viral vector. Any adenovirus (Ad) vector system that will promote expression of iNOS in the target cell of interest may be utilized. Any number of eukaryotic promoters available to one of ordinary skill in the art may be used in constructing an adenovirus-iNOS gene therapy vector. Therefore, any eukaryotic promoter and/or enhancer sequences available to the skilled artisan which are known to control expression of the nucleic acid of interest may be used in Ad vector constructs, including but not limited to a cytomegalovirus (CMV) promoter, a Rous Sarcoma (RSV) promoter, a Murine Leukemia (MLV) promoter, a β -actin promoter, as well as any additional tissue specific or signal specific regulatory sequence that induces expression in the target cell or tissue of interest. Adenovirus gene therapy vectors will be advantageous due to, for example, (1) efficient infection of nondividing cells such as endothelial cells and hepatocytes and, (2) the transient nature of adenovirus vector expression in the target cell, which will be advantageous in applications to prevent thrombosis immediately post-angioplasty.

In an additional embodiment of the invention, a DNA sequence encoding iNOS or a biologically active fragment thereof may be subcloned into an adeno-associated viral vector (AAV). One of ordinary skill in the art may construct a recombinant AAV-iNOS vector to be utilized in any one of a number of gene therapy applications. In contrast to

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retroviral terminal repeat sequences, AAV terminal repeat sequences do not contain regulatory sequences which promote foreign gene expression. As discussed above for Ad vectors, any eukaryotic promoter and/or enhancer sequences available to the skilled artisan which are known to control expression of the nucleic acid of interest may be used
5 in AAV vector constructs, including but not limited to a cytomegalovirus (CMV) promoter, a Rous Sarcoma (RSV) promoter, a Murine Leukemia (MLV) promoter, a β -actin promoter, as well as any additional tissue specific or signal specific regulatory sequence that induces expression in the target cell or tissue of interest.

An appropriate recombinant AAV-iNOS vector can be utilized to directly infect
10 *in vitro* cultured endothelial cells or vascular smooth muscle cells. Endothelial cells infected with recombinant AAV-iNOS can then be delivered to the specific tissue target site utilizing methods known in the art, including but not limited to the catheterization techniques disclosed within this specification. Alternatively, recombinant AAV-iNOS can be delivered to the target cell through association with liposome microcapsules. A
15 transfection protocol utilizing a hybrid liposome:AAV construct involves using an AAV vector (most likely with both LTR's present) comprising an iNOS DNA sequence. This construct is cotransfected into target endothelial cells or vascular smooth muscle cells with a plasmid containing the *rep* gene of AAV. Transient expression of the *rep* protein enhances stable integration of the recombinant AAV-iNOS genome into the endothelial
20 cell or vascular smooth muscle cell genome.

In addition to the hereinbefore described use of viral vectors to infect target cells, any known non-viral vector that is capable of expression upon transfection of a specified eukaryotic target cell may be utilized to practice the present invention. Such non-viral based vectors include, but are not solely limited to, plasmid DNA.

One of ordinary skill in the art will be guided by the literature to choose an
25 appropriate DNA plasmid vector for use in the present invention. As discussed above for recombinant Ad and AAV vectors, any eukaryotic promoter and/or enhancer sequences available to the skilled artisan which are known to control expression of the nucleic acid of interest may be used in plasmid vector constructs, including but not limited to a to
30 cytomegalovirus (CMV) promoter, a Rous Sarcoma (RSV) promoter, a Murine Leukemia (MLV) promoter, a herpes simplex virus (HSV) promoter, such as HSV-tk, a β -actin promoter, as well as any additional tissue specific or signal specific regulatory sequence

that induces expression in the target cell or tissue of interest. A signal specific promoter fragment includes but is not limited to a promoter fragment responsive to TNF.

In one such embodiment, a DNA sequence encoding human iNOS is subcloned into the DNA plasmid expression vector, pCIS (Genentech), resulting in pCIS-iNOS.

5 pCIS is a standard mammalian expression vector, containing an antibiotic resistance gene for propagation in *E. coli* and a CMV promoter active in mammalian cells. Such a construct, which may be constructed by one of ordinary skill with components available from numerous sources, will drive expression of an iNOS DNA fragment ligated downstream of the CMV promoter subsequent to transfection of the target cell. More
10 specifically, a NotI/XhoI restriction fragment containing the human iNOS coding region is generated and isolated from pHINOS (pHINOS is deposited with the ATCC with accession number 75358) and ligated into NotI/XhoI digested pCIS. Alternatively, the isolated human iNOS sequence may be fused to any portion of the wild type human iNOS promoter sequence such that expression of human iNOS can be induced within the target
15 cell.

In a preferred embodiment utilizing plasmid DNA to transfect target cells, a plasmid vector comprising a DNA sequence encoding iNOS or a biologically active fragment thereof will be utilized in liposome-mediated transfection of the target cell choice as described within this specification. The stability of liposomes, coupled with the
20 impermeable nature of these vesicles, makes them useful vehicles for the delivery of therapeutic DNA sequences (for a review, see Mannino and Gould-Forgerite, 1988, BioTechniques 6(7): 682-690). Liposomes are known to be absorbed by many cell types by fusion. In one embodiment, a cationic liposome containing cationic cholesterol derivatives, such as SF-chol or DC-chol, may be utilized. The DC-chol molecule
25 includes a tertiary amino group, a medium length spacer arm and a carbamoyl linker bond as described by Gao and Huang (1991, Biochem. Biophys. Res. Comm. 179: 280-285). As an example, but not a limitation, the pCIS-iNOS plasmid construction can be utilized in liposome-mediated *in vitro* transfection of cultured endothelial cells as well as *in situ* transfection of endothelial cells.

30 In another embodiment regarding the use of liposome technology, the viral or nonviral based vector comprising the DNA sequence encoding a biologically active iNOS protein fragment is delivered to the target cell by transfection of the target cell with

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lipofectamine (Bethesda Research Laboratory). Lipofectamine is a 3:1 Liposome formulation of the polycationic lipid 2,3 dioleoyloxy -N- [2(sperminecarboxymido)ethyl]-N,N-dimethyl-1- propanaminiumtrifluoroacetate (DOPSA) and the neutral lipid dioleoyl-phosphatidylethanolamine (DOPE).

5 Other uses of non-viral modes of gene delivery include, but are not limited to, (a) direct injection of naked DNA; (b) calcium phosphate $[Ca_3(PO_4)_2]$ mediated cell transfection; (c) mammalian host cell transfection by electroporation; (d) DEAE-dextran mediated cell transfection; (e) polybrene mediated delivery; (f) protoplast fusion; (g) microinjection; and (h) polylysine mediated transformation and the genetically
10 engineered cells transferred back to the mammalian host.

The present specification discloses preferred methods of gene therapy-based increase in local human iNOS expression within a targeted region of an artery or within a synthetic conduit utilized to bypass a diseased segment of the arterial vessel.

For example, a preferred method involves *in vitro* targeting of cultured endothelial
15 cells, vascular smooth muscle cells or a combination of both cell types with a human iNOS DNA fragment ligated into a retroviral vector, such as MFG. A preferred retroviral construct is MFG-iNOS. Such a retroviral vector is transfected into an appropriate packaging cell line to generate infectious virus which is then used to infect
20 *in vitro*. Once a diseased arterial vessel is substantially cleared of a stenosis or occlusion, the iNOS-infected endothelial and/or vascular smooth muscle cells may be used to repopulate a region of the diseased arterial wall. It will be known to the skilled vascular surgeon that various endovascular surgical techniques are available, depending upon the severity of the occlusion and location of the diseased arterial vessel (for a review, see
25 generally Ahn, 1993, "Endovascular Surgery", in Vascular Surgery, A Comprehensive Review, Ed. W.S. Moore, W.B. Saunders & Co., Philadelphia). For example, such endovascular surgical procedures include but are not limited to balloon angioplasty, laser-assisted balloon angioplasty, double balloon catheterization, mechanical endarterectomy and vascular endoscopy. In addition, a preferred means of physically
30 separating the diseased area of the arterial vessel for delivery of iNOS-expressing cells would be the use of a double balloon catheter.

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Preferred modes of *in vitro* infection of arterial luminal cells include human iNOS-containing recombinant retrovirus, especially MFG-iNOS; liposome-mediated transfection of a recombinant iNOS-containing plasmid vector, especially pCIS-iNOS, a recombinant adenovirus vector or a recombinant adeno-associated virus vector. It will be understood to the skilled artisan that similar *in vitro* infection or transfection procedures may be utilized whether the target cell is an endothelial cell or a vascular smooth muscle cell.

An additional method directing increased local iNOS expression at specific sites within an artery involves iNOS-containing recombinant viral infection of endothelial cells, vascular smooth muscle cells or a combination of both *in situ*. As discussed in the previous paragraph, this method involves substantially clearing a stenosis or occluded region of said arterial vessel and physically segregating the cleared region of said arterial vessel so as to act as a receptacle for recombinant iNOS viral particles. Again, the preferred manner of clearing a stenosis include but are not limited to balloon angioplasty, laser-assisted balloon angioplasty, mechanical endarterectomy and vascular endoscopy. Additionally, a preferred means of physically separating the manipulated segment of the arterial vessel for delivery of recombinant iNOS-viral particles would be the use of a double balloon catheter.

Preferred modes of *in situ* infection of arterial luminal cells include iNOS-containing recombinant viral particles, especially MFG-iNOS; liposome-mediated transfection of a recombinant iNOS-containing plasmid vector, especially pCIS-iNOS, a recombinant adenovirus vector or a recombinant adeno-associated virus vector. Both endothelial and vascular smooth muscle cells may be infected or transfected simultaneously through *in situ* procedures, exemplified but not limited to the *in situ* procedure outlined in Example Section 13.1.2.

Additional preferred methods of iNOS based gene therapy treatment of vascular disease involves vascular surgery. More specifically, vascular surgical procedures characterized by:

(1) Infecting or transfecting *in vitro* cultured mammalian cells selected from the group consisting of endothelial cells, vascular smooth muscle cells or a combination of both cell types with a human iNOS-containing viral or non-viral vector encoding a biologically active iNOS protein or protein fragment; seeding a synthetic or autogenous

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conduit with a population of the human iNOS-transfected cells; and forming a proximal and a distal anastomosis which bypass a diseased arterial vessel segment within said patient. iNOS-based gene therapy combined with vascular bypass techniques will promote expression of iNOS within the graft, resulting in prophylactic and therapeutic relief by preventing or substantially reducing intimal hyperplasia, thrombogenicity, and other forms of post-operative occlusive complications which commonly occur following vascular bypass procedures.

(2) Infecting or transfecting *in vitro* cultured endothelial cells, vascular smooth muscle cells or a combination of both with recombinant human iNOS; forming a proximal and a distal anastomosis to bypass a diseased portion of an arterial vessel within said patient; physically segregating each anastomosis subsequent to graft suturing; and seeding the isolated area at and around the distal and the proximal anastomoses with arterial cells infected or transfected with a human iNOS construct to promote increased local iNOS expression within the proximity of the anastomoses.

(3) Forming a proximal and a distal anastomosis to bypass a diseased portion of an arterial vessel within the patient; physically isolating each said anastomosis subsequent to graft suturing; and transfecting cells *in situ* (endothelial, smooth muscle or both) which line the arterial lumen around the target anastomosis with a human iNOS construct such that localized expression of iNOS imparts prophylactic and therapeutic relief from said human vascular disease and from the development of intimal hyperplasia.

(4) Surgically opening an arterial vessel at a site of luminal narrowing or occlusion and performing endarterectomy to reestablish patency; following closure of this site of repair, this site of surgical injury can be seeded with cultured endothelial cells or vascular smooth muscle cells carrying a human iNOS construct to increase local iNOS expression to prevent reocclusion.

(5) Surgically opening an arterial vessel at a site of luminal narrowing or occlusion and performing endarterectomy to reestablish patency; following closure of this site of repair, this site of surgical injury can be seeded by any of the *in situ* methods disclosed in this specification or any other *in situ* technique available to the skilled artisan.

The preferred means of physically separating the proximal and/or distal anastomosis will be use of a double balloon catheter.

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The preferred means of seeding vascular grafts include endothelial and vascular smooth muscle cells infected or transfected with iNOS-containing recombinant viral particles, preferably a recombinant retroviral particle and especially an MoMLV retroviral particle such as MFG-iNOS; liposome-mediated transfection of a recombinant iNOS-construct, especially pCIS-iNOS, and adenovirus or adeno-associated virus based vector iNOS constructs (either directly as a viral supernatant or via liposome-mediated transfection of the arterial cells).

This specification discloses to the skilled artisan use of any conduit available to the vascular surgeon in classical bypass procedures in the iNOS-based gene therapy procedures described herein. The present invention envisions the use of numerous conduits, including but not limited to venous autografts, (especially the saphenous vein), synthetic grafts (especially polytetrafluoroethylene [PTFE]), arterial autografts, umbilical vein autografts, and xenografts.

5.2.2. BIOLOGIC THERAPY BY PROMOTION OF ANTITUMOR EFFECTS

The L-arginine:NO pathway has been shown to be involved in antitumor activity (Hibbs, et al., 1987, Science 235: 473-476; Kilbourn, et al., 1990, Proc. Natl. Acad. Sci. USA 87: 3629-3632). The biological activity of nitric oxide is thought to include inhibition of DNA synthesis and mitochondrial enzymes involved in respiration.

The present invention discloses methods of human iNOS-directed gene therapy to promote antitumor effects in cancer patients. Such a human iNOS-directed cancer gene therapy will provide a local increase in nitric oxide concentration within the area of the tumor to be treated, thus promoting antitumor activity without systemic increases in nitric oxide levels.

Therefore, the present invention discloses targeting of a DNA sequence to specific sites within a patient such that local expression of iNOS will lead to increased nitric oxide concentration, thus stimulating antitumor activity.

The isolated human iNOS DNA sequence may be manipulated *in vitro* in a number of ways available to one of ordinary skill in the art so as to promote local expression of recombinant iNOS or a biologically active fragment thereof.

The human iNOS DNA sequence encoding the intact iNOS protein or a partial DNA sequence thereof encoding a biologically active fragment thereof will be delivered

to the target cell by *in vitro* transduction utilizing the viral and non-viral methods discussed in Section 5.2.1. The *in vitro* transduced target cell is then introduced into the patient so as to promote local iNOS expression at the tumor site. Therefore, it will be understood that any human iNOS DNA sequence, regardless of tissue source, is a candidate for cancer gene therapy. Such an iNOS DNA sequence may include, but is not limited to, (1) an isolated cDNA or genomic sequence purified from human hepatocyte cells, or a DNA sequence from said source which encodes a biologically active fragment of human iNOS; or (2) an isolated cDNA or genomic fragment purified from a human non-hepatocyte source, or a DNA sequence from said source which encodes a biologically active fragment of human iNOS.

Any of the above-identified iNOS sequences may be fused to a tissue specific or signal specific promoter fragment active within the target cell, or alternatively, may be fused to the wild type human iNOS promoter sequence. An example of a signal specific promoter in iNOS-driven biologic therapy applications would include, but is not limited to, a promoter upregulated in response to TNF. Therefore, any promoter or enhancer sequence which increases the local expression of iNOS within the transformed target cell is a candidate for use in antitumor applications.

Promotion of local expression of iNOS at or around the tumor site is dependent on utilizing an appropriate target cell for *in vitro* transduction and introduction into the patient. In one embodiment regarding cancer gene therapy, the patient is intravenously injected with *in vitro* transduced target cells, including but not limited to tumor infiltrating lymphocytes originally harvested from the patient.

The delivery to the target cell may be accomplished by viral or non-viral methods primarily as described in Section 5.2.1. These methods include, but are not limited to (a) retroviral vectors, including but not limited to vectors derived from Moloney murine leukemia virus (MoMLV); (b) adeno-associated vectors; (c) adenovirus vectors; (d) herpes simplex virus vectors; (e) SV40 vectors; (f) polyoma virus vectors; (g) papilloma virus vectors; (h) picornavirus vectors; and (i) vaccinia virus vectors. Depending on the vector system chosen, techniques available to the skilled artisan are utilized to infect the target cell of choice with the recombinant virus vector.

In a preferred method of delivering a human iNOS sequence to the target cell of interest, a recombinant retroviral vector carrying a DNA sequence encoding iNOS or a

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biologically active fragment thereof is utilized to infect tumor infiltrating lymphocytes. These infected tumor infiltrating lymphocytes are then reintroduced into the patient to promote local production of nitric oxide at the tumor site.

Any number of retroviral constructs which express a biologically active form of iNOS may be utilized to promote antitumor activity. Preferably, MFG-iNOS or DFG-iNOS-Neo may be utilized to infect cultured tumor infiltrating lymphocytes or tumor cells harvested from the patient.

One of ordinary skill in the art will understand that any additional isolated DNA sequence encoding a biologically active portion of iNOS, as hereinbefore disclosed, may be subcloned into a retroviral vector for eventual *in vitro* infection of cultured tumor infiltrating lymphocytes or tumor cells harvested from the patient.

In addition to the hereinbefore described use of viral vectors to infect target cells, any known non-viral vector that is capable of expression upon transfection of a specified eukaryotic target cell may be utilized to practice the present invention. Such non-viral vectors include, but are not solely limited to, plasmid DNA.

One of ordinary skill in the art will be guided by the literature to choose an appropriate plasmid vector for use in the present invention. Any eukaryotic promoter and/or enhancer sequence available to the skilled artisan which is known to control expression of the nucleic acid of interest may be used in plasmid vector constructs, including but not limited to a cytomegalovirus (CMV) promoter, a Rous Sarcoma (RSV) promoter, a Murine Leukemia (MLV) promoter, a β -actin promoter, as well as any additional tissue specific or signal specific regulatory sequence that induces expression in the target cell or tissue of interest. In a specific embodiment of the invention, the plasmid vector comprising an iNOS DNA sequence is pCIS-iNOS.

Delivery of iNOS-plasmid constructs to a target cell type, such as tumor cells, may be accomplished by numerous biologic and non-biologic carriers available to one of ordinary skill in the art. In a preferred embodiment utilizing plasmid DNA to transfect target cells, a plasmid vector comprising a DNA sequence encoding iNOS or a biologically active fragment thereof will be utilized in liposome-mediated transfection, as described in detail in Section 5.2.1.

Other uses of non-viral modes of gene delivery include, but are not limited to, (a) direct injection of naked DNA; (b) calcium phosphate [$\text{Ca}_3(\text{PO}_4)_2$] mediated cell

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transfection; (c) mammalian host cell transfection by electroporation; (d) DEAE-dextran mediated cell transfection; (e) polybrene mediated delivery; (f) protoplast fusion; (g) microinjection; and (h) polylysine mediated transformation; and the genetically transformed cells then transferred back to the mammalian host.

5

5.2.3. BIOLOGIC THERAPY FOR TREATING MICROBIAL INFECTIONS

The human iNOS DNA sequences of the present invention may be utilized in treating microbial infections. Specifically, iNOS-driven biologic therapy will be utilized to treat microbes known to be susceptible to increased concentrations of nitric oxide.

10 Nitric oxide is known to be a cytotoxic effector molecule against mycobacteria, helminths, fungi protozoa, and viruses.

Upon review of this specification, the artisan of ordinary skill will be directed to utilize any of the iNOS sequences listed in Section 5.2.1. iNOS-driven antimicrobial therapy is dependant on targeting the respective human iNOS sequence to the
15 tissue-specific cell type harboring the microbe or to the microbe itself. Depending upon the targeted microbe or cell type, delivery of the human iNOS DNA may be accomplished by biologic or non-biologic means.

In a preferred embodiment of utilizing iNOS-driven antimicrobial therapy, the target cells are human hepatocytes infected with the sporozoa *Plasmodium*, the causative
20 agent of malaria. Human malaria is caused by one of four species of *Plasmodium*: *P. falciparum*, *P. malariae*, *P. vivax* and *P. ovale*. The sporocytes of *Plasmodium* penetrate hepatocyte cells subsequent to entry into the circulatory system of the human host.

In a preferred embodiment of treating malaria via iNOS-driven biologic therapy,
25 the iNOS-vector is delivered via liposome mediated transformation of the target hepatocytes.

In another embodiment of the invention, iNOS driven antimicrobial therapy is utilized to treat helminthic infections, including but not solely limited to Schistosomiasis (e.g., *Schistosoma mansoni*, *Schistosoma haematobium*, and *Schistosoma japonicum*.
30 Direct treatment of helminth infected liver cells encompass all techniques described above for iNOS driven therapy of malaria.

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In an especially preferred embodiment of treating malaria via iNOS-driven antimicrobial therapy, the liposomes are modified by insertion of an hepatocyte specific asialoprotein into the liposome complex. The resulting asialoprotein binds to the galactose receptor unique to hepatocytes (see Wall, et. al., 1980, Cell 21: 79-83).

5 Therefore, encapsulating the iNOS DNA vector within an asialoprotein-containing liposome will direct delivery specifically to hepatocytes.

Another embodiment of utilizing iNOS-vectors in antimicrobial therapy involves treatment of lung borne microbial infections, including but not limited to tuberculosis and leprosy.

10 The causative agent of tuberculosis is *Mycobacterium tuberculosis*, which enters the lung via droplet nuclei and the respiratory route. Once in the lungs, this bacterium grows and eventually is surrounded by lymphocytes, macrophages and connective tissue, forming nodules called tubercles. Normally, this represents the end stage of the infection, with no ill effects. Alternatively, a caseous lesion may form, which may
15 calcify to form a Ghon complex and further become liquified, forming tuberculous cavities.

A preferred treatment of tuberculosis by iNOS-driven antimicrobial therapy involves directing an iNOS vector to the target tissue by viral mediated transformation of cells within the target tissue.

20 One preferred method of viral mediated delivery is retroviral mediated delivery, as discussed in Section 5.2.1. With the aid of this specification, it is within the realm of the artisan of ordinary skill to construct an iNOS vector for use in treating tuberculosis.

Another preferred method of viral mediated delivery is adenovirus mediated delivery, wherein the iNOS DNA fragment of interest is inserted into an adenovirus
25 vector.

A preferred method of administering iNOS-retroviral or iNOS-adenoviral vectors to invected regions of lung is inhalational administration, in the form of an aerosol mist.

The target would be advanced disseminated disease including but not limited to the treatment of tuberculosis, as well as other microbial infections such as fungal infections in
30 a transplant patient, and disseminated aspergillosis or fungal or additional viral infections such as cytomegalovirus in an AIDS patient.

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The causative agent of leprosy is *Mycobacterium leprae*. Transmission of leprosy is highest when children are exposed to infected individuals shedding *M. leprae*. Nasal secretions are the most likely infectious material within family contacts. The preferred mode of iNOS viral delivery is through inhalational administration, as described for

5 *M. tuberculosis*, is also the preferred mode of treating *M. leprae*.

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6. EXAMPLE: INDUCING HUMAN HEPATOCYTE INDUCIBLE NITRIC OXIDE SYNTHASE

iNOS mRNA is weakly induced in hepatocytes following stimulation with individual cytokines such as for example tumor necrosis factor (TNF), interleukin-1 (IL-1) or interleukin-gamma (IFN-g). Cytokines can synergize to further up-regulate iNOS mRNA levels and nitric oxide synthase activity. Maximum induction of iNOS was achieved with a combination of TNF, IL-1, IFN-g and bacterial lipopolysaccharide (LPS). (Geller, et al., 1993, Proc. Natl. Acad. Sci. 90: 522-526; Nussler, et al., 1992, J. Exp. Med. 176:261-264).

7. EXAMPLE: IDENTIFYING AND ISOLATING HUMAN HEPATOCYTE NITRIC OXIDE SYNTHASE mRNA

A cross-species iNOS cDNA probe capable of hybridizing with human hepatocyte inducible nitric oxide synthase mRNA was used to identify and isolate the mRNA for human hepatocyte inducible nitric oxide synthase. The time-point of peak iNOS mRNA levels following cytokine and LPS [hereinafter cytokine mixture (CM)] stimulation was then determined.

Total cellular RNA was extracted 2-48 hours following CM-stimulation of cultured human hepatocytes using the RNazol B modified method of Chomczynski and Sacchi (1987, Anal. Biochem. 162:156-159). 20 microgram aliquots of total RNA were examined by Northern blot analysis through cross-species hybridization with a murine macrophage iNOS cDNA probe generated from a fragment of the murine iNOS cDNA isolated after NotI restriction enzyme digest. (Lowenstein, et al., 1992, Proc. Natl. Acad. Sci. USA. 89:6711-6715; GenBank Accession No. M92649). The presence of human hepatocyte nitric oxide synthase mRNA was identified as a single band of about 4.5 Kb size with maximal iNOS mRNA levels seen about 8 hours after CM stimulation.

Figure 2 shows the presence of the 4.5 kb message for human hepatocyte inducible nitric oxide synthase. Freshly isolated human hepatocytes (HC) were placed in cell culture and exposed to a combination of human recombinant tumor necrosis factor (500 units/milliliter), human recombinant interleukin-1 (5 units/milliliter), human recombinant interferon-gamma (100 units/milliliter), and lipopolysaccharide (10 micrograms/milliliter). Figure 2 shows that total RNA was isolated at the indicated time points (2, 4, 6, & 8 hrs.) and 20 micrograms per sample was subjected to Northern blot analysis. A 2.7 Kb

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cDNA fragments for murine macrophage inducible nitric oxide synthase was used to identify the human hepatocyte inducible nitric oxide synthase mRNA. Figure 2 demonstrates that the 4.5 Kb message level peaked at about 8 hours following stimulation. Figure 2 shows that no mRNA signal was detected in control (unstimulated) hepatocytes. Figure 3 shows the expression of the 4.5 Kb human hepatocyte inducible nitric oxide synthase mRNA at about 8 hours after exposure to the above mentioned cytokines from hepatocytes isolated from three separate individuals [patent (Pt.) 1, 2, and 3]. Figure 3 demonstrates that no signal was detected in control (unstimulated) hepatocytes.

Because the 8 hour time point yielded maximal iNOS mRNA levels, total cellular RNA was isolated from two human livers about 8 hours following CM-stimulation *in vitro*. cDNA synthesis requires about 10 to 20 micrograms of poly A mRNA rather than total RNA. Poly A mRNA was purified from total cellular RNA by elution through an oligo-dT cellulose column. To identify the presence of human hepatocyte iNOS mRNA in the purified poly A mRNA, repeat Northern blot analysis was performed on 0.5 micrograms of purified A mRNA from each of the two human livers using the 2.7 Kb cDNA probe for murine macrophage inducible nitric oxide synthase. Figure 4 shows strong nitric oxide synthase mRNA bands from the 2 different patients without evidence of degraded poly A RNA.

Figure 4 shows that the murine macrophage inducible nitric oxide synthase cDNA probe effectively cross hybridizes and identifies the human hepatocyte inducible nitric oxide synthase mRNA in the poly A RNA. The samples of poly A mRNA from the 2 patients were pooled and were used to construct the cDNA library for isolation of cDNA clone for the human hepatocyte inducible nitric oxide synthase.

8. EXAMPLE: CONSTRUCTING A HUMAN HEPATOCYTE INDUCIBLE NITRIC OXIDE SYNTHASE cDNA LIBRARY

Using about 20 micrograms of the poly A mRNA isolated from CM-stimulated human hepatocytes, a cDNA library was constructed by Stratagene, La Jolla, CA. The first strand cDNA was synthesized from the human hepatocyte poly A mRNA using MoMLV reverse transcriptase enzyme with oligo-dT primers. After excluding strands less than 1000 nucleotide basis pairs in length, the cDNA's were inserted into a lambda Zap II phage vector (Stratagene, La Jolla, CA) and was titered.

9. EXAMPLE: SCREENING THE cDNA LIBRARY FOR HUMAN HEPATOCYTE INDUCIBLE NITRIC OXIDE SYNTHASE cDNA CLONES

To screen the cDNA library, 1×10^6 phage were incubated with bacteria (*E. coli* Sure strain) at 34 to 40 degrees centigrade for 15 to 30 minutes. This mixture was added to molten agarose and poured onto 20 x 20 centimeter agar plates at a density of 2×10^5 plaques/plate (Maniatis et al., 1982, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY,). The plates were incubated at 34 to 40 degrees centigrade overnight for 6 to 24 hours to allow for phage lysis of bacteria. The plaques were then transferred to nitrocellulose filters and clones carrying iNOS cDNA inserts were identified by filter hybridization with ^{32}P -labeled murine macrophage inducible nitric oxide synthase cDNA probe. Positively labeled clones were cored from the agar plates after localization by autoradiograph alignment. The positive clones were rescued from the lambda Zap II phage vector with the helper phage ExAssist (Stratagene, La Jolla, CA), and then converted to plasmid vectors using pBluescript (Stratagene, La Jolla, CA). The cDNAs for human hepatocyte inducible nitric oxide synthase were excised from the Bluescript plasmid cloning sites by restriction with EcoRI enzyme and then sized by gel electrophoresis to identify a full-length clone. The cDNA identities were confirmed by DNA sequencing and by Southern blot hybridization with the murine macrophage iNOS cDNA probe. In addition, Northern blot analysis of cytokine-stimulated human hepatocyte poly A mRNA was performed using the full-length human inducible nitric oxide synthase cDNA clone of this invention as the probe. Figure 5 shows a time course for the expression of human hepatocyte inducible nitric oxide synthase mRNA. This RNA was isolated from a patient different from the patients listed in Figures 2 and 3. The cells of the patient in Figure 5 were exposed to the same agents as described for Figure 2. Figure 5 shows the cloned human inducible nitric oxide synthase cDNA identifies the same size mRNA signal as the murine macrophage iNOS cDNA probe, thus, further confirming its identify. It is important to note that the isolated cDNA clone coding for human inducible nitric oxide synthase of this invention can hybridize with human inducible nitric oxide synthase mRNA, thus, confirming the capacity of the cDNA clone of this invention to identify the human hepatocyte inducible nitric oxide synthase mRNA.

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10. EXAMPLE: cDNA SEQUENCING

The plasmid vector pBluescript contains universal primer regions which were used to facilitate double-stranded DNA sequencing. Positive clones were sequenced by using the dideoxynucleotide technique of Sanger, supra, with the Genesis 2000 sequencing system (USB, Cleveland, Ohio). Sequence analysis was done using Genbank DNA sequencing software programs available through the Pittsburgh Supercomputing Center (Billiar TR., Pittsburgh Supercomputing Center, Pittsburgh, PA).

11. EXAMPLE: EXPRESSING HUMAN HEPATOCYTE
INDUCIBLE NITRIC OXIDE SYNTHASE

Verification of the full length cDNA identity was accomplished by expressing the recombinant human hepatocyte inducible nitric oxide synthase protein. The human hepatocyte inducible nitric oxide synthase cDNA was ligated into the pCIS expression vector (Genentech, CA) which utilizes a CMV promoter. Next the expression vector was transfected into human embryonic kidney 293 cells (ATCC, Maryland). Nitric oxide synthase activity was assessed by measuring the conversion of [^3H] arginine to [^3H] citrulline. It will be appreciated by those skilled in the art that this expression system was successfully used for expression of the cloned rat brain constitutive nitric oxide synthase, and there was negligible nitric oxide synthase activity in the unstimulated 293 kidney cells (Bredt et al., 1991, Nature, 351:714-718). After the identity of the human hepatocyte inducible nitric oxide synthase cDNA clone of this invention was verified as hereinbefore described, the cDNA was expressed in a baculovirus expression system (Invitrogen, San Diego, CA) which allowed for large scale enzyme production (1988, Texas Agriculture Experiment Station Bulletin, No. 1555). More specifically, the human hepatocyte nitric oxide synthase cDNA was inserted into the baculovirus transfer vector and then co-transfected with wild type viral DNA into Sf9 insect cells (ATCC, Maryland). Recombinant viral plaques were isolated to allow for protein over-expression.

12. EXAMPLE: PURIFYING THE HUMAN HEPATOCYTE
INDUCIBLE NITRIC OXIDE SYNTHASE PROTEIN

The resultant human hepatocyte inducible nitric synthase protein was purified using a two step procedure. First, the protein was passed through an anion-exchange column of DEAE cellulose. This was followed by affinity chromatography with 2', 5'-

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ADP Sepharose. (Evans et al., 1992, Proc. Natl. Acad. Sci. USA, 89:5361-5365).

Purity was assessed by SDS-polyacrylamide gel electrophoresis. Activity was quantitated after each step by measuring the ability of the enzyme to generate NO_2^- and NO_3^- from L-arginine. NO_2^- and NO_3^- was measured using an automated colorimetric reaction based on the Green reaction (Green, et al., 1982, Anal. Biochem. 126:131-137)

Whereas, particular embodiments of this invention have been described above for purposes of illustration, it will be evident to those persons skilled in the art that numerous variations of the details of the present invention may be made without departing from the invention as defined in the appended claims that follow the SEQUENCE LISTING.

13. EXAMPLE: TREATMENT OF VASCULAR OCCLUSIVE DISEASE

13.1. MATERIALS AND METHODS

Total RNA extraction, Northern, Southern, Western blots, and PCR are techniques routinely performed by one of ordinary skill in the art (e.g., see generally Maniatis, et al., 1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY; Geller, et al., Proc. Natl. Acad. Sci. USA 90: 522-526; Towbin, et al., 1979, Proc. Natl. Acad. Sci. USA 76: 4350; Brenner, et al., 1989, BioTechniques 7: 1096-1103). The direct iNOS enzyme assay measures the conversion of $[\text{^3H}]$ -arginine to $[\text{^3H}]$ -citrulline, as described (Bredt, et al., 1991, Nature 351:714-718). $\text{NO}_2^- + \text{NO}_3^-$ levels are measured in the culture supernatants by an automated procedure based on the Griess reaction (Green, et al., 1982, Anal. Biochem. 126: 131-137).

Briefly, immunohistochemistry for iNOS is performed as follows: The cells are washed twice in phosphate buffered saline (PBS), fixed in 2% paraformaldehyde in PBS, and permeabilized with 2% paraformaldehyde in PBS containing 0.1% Triton X100. Cells are then incubated for 2 hours with the primary antibody. AntiNOS antibody labeled specimens are then washed and incubated with a biotinylated secondary antibody, washed again and treated with 0.1% hydrogen peroxide. Following additional washes the cells are treated with ABC reagent for 45 minutes, washed, reacted with diaminobenzidine for 5 minutes, and finally washed with H_2O prior to counter staining with toluidine blue.

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13.1.1. IN VITRO TRANSFECTION OF TARGET CELLS WITH A HUMAN iNOS cDNA

Primary porcine endothelial cells, derived from the Yucatan minipig (YPE cells), will be isolated as described by Reitman, et al. (1982, Atherosclerosis 43: 119-132) as outlined in Nabel, et al. (1989, Science 244: 1342-1344). Cells are incubated with medium 199 (M199) supplemented with 10% FBS, 2 mM L-glutamine, 50 units/ml penicillin, and 5 ug/ml streptomycin.

Gene transfer to endothelial cells by retroviral-based infection, adeno-associated viral-based infection as well as liposome mediated transfection will be utilized. To exemplify retroviral infection, the retroviral vector is an MFG vector which is utilized to construct MFG-iNOS (see Figure 6 and Figure 7). The MFG vector is a simplified MoMVL vector in which the DNA sequences encoding the *pol* and *env* proteins have been deleted so as to render it replication defective. The majority of the *gag* sequence has also been deleted. The human hepatocyte iNOS cDNA was inserted into the NcoI and BamHI cloning sites of the retroviral vector MFG as shown in Figure 6 and Figure 7. Briefly, the MFG vector has a unique cloning region consisting of a 5' NcoI site and a 3' BamHI site. PCR primers were used to generate a point mutation at bp 205 of the iNOS cDNA, manufacturing an NcoI site that incorporated the ATG start codon. A 5' fragment of the PCR product of the iNOS cDNA spanning from the NcoI site at bp 205 to the EcoRI site at bp 1059 was isolated. The 3' BamHI site was generated by linearizing the pBScript-iNOS plasmid with AflII which uniquely cut at bp 3705 of the iNOS cDNA. This restriction site is located approximately 40 bp downstream from the iNOS stop codon. A BclI linker was then ligated to the linearized plasmid. Double digestion with EcoRI and BclI allowed the isolation of a 3' fragment of the iNOS cDNA from bp 1060 (EcoRI) to bp 3710 (BclI). The BclI overhang is complementary to the overhang generated by BamHI. A three part ligation was then performed between MFG, the 5' PCR product with the 5' NcoI site, and the 3' fragment with the 3' BclI linker. *Escherichia coli* were transformed with the ligation mixture and grown on ampicillin selection. Transformants were isolated and screened for the properly reconstituted MFG-iNOS construct. One correct transformant was isolated and large scale plasmid DNA preparations performed.

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A second retroviral vector construct was also made, this one to include a selectable neomycin resistance marker (see Figure 6 and Figure 7.) The MFG retroviral vector had been previously engineered to contain an internal ribosome entry site (IRES) followed by a neomycin resistance gene (Neo^R) inserted at the 3' BamHI cloning site of MFG. The IRES sequence allows for the translation of multiple protein products from a single polycistronic mRNA. This MFG-IRES-Neo^R plasmid was digested with the restriction enzymes SalI (which cuts approximately 3000 bps upstream of the NcoI cloning site of MFG) and Bam HI. The larger fragment containing the majority of the MFG backbone attached to IRES and Neo^R was purified. The previously constructed MFG-iNOS vector was also digested with SalI and EcoRI and a 3.7 Kb fragment containing the 5' portion of the iNOS cDNA was isolated. The 3' end of the iNOS cDNA was the identical 3' fragment with the BclI linker used to construct MFG-iNOS. A 3 part ligation with MFG-IRES-Neo^R, 5' SalI - EcoRI fragment containing the 5' end of the iNOS cDNA, and 3' iNOS cDNA with the BclI linker was performed. The ligation mixture was then transformed into *E. coli* and selected for ampicillin resistant transformants. Such a positive transformant with the correctly oriented construct, referred to throughout this specification as DFG-iNOS-Neo^R, was isolated and large scale plasmid preparation performed.

These retrovirus vectors carrying a portion of the human hepatocyte iNOS cDNA are transfected into the CRIP cell packaging line (Danos and Mulligan, 1988, Proc. Natl. Acad. Sci. USA 85: 6460-6464) using a standard calcium phosphate transfection procedure. The viral vector DFG-iNOS-Neo^R is capable of imparting resistance to the synthetic antibiotic G418. CRIP cells transfected with DFG-iNOS-Neo were selected on the basis of resistance to G418. The CRIP cell line expresses the three viral proteins required for packaging the recombinant viral RNAs into infectious particles. Moreover, the viral particles produced by the CRIP cell line are able to efficiently infect a wide variety of species of mammalian cells including human cells. All retroviral particles produced by this cell line are defective for replication but retain the ability to stably integrate into mammalian cells, thereby transferring an heritable trait to these cells. Virus stocks produced by this method are substantially free of contaminating helper-virus particles and are also non-pathogenic.

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Viral supernatants for both the MFG-iNOS and DFG-iNOS-Neo^R vectors were used to infect endothelial cells *in vitro* as described by Zwiebel, et al. (1989, Science 249:220-222). Briefly, CRIP producer cells transfected with MFG-iNOS proviral DNA carried no selectable markers. A heterogenous population of CRIP cells, some producing MFG-iNOS virus and some not, was grown and viral supernatants collected to infect endothelial cells. Those CRIP cells transfected with DFG-iNOS-Neo^R carried a selectable marker (resistance to G418) and a CRIP producer line generating high titer MFG iNOS-Neo viral supernatants was isolated and propagated. Viral supernatants for MFG-iNOS and DFG iNOS-Neo^R were found to be free of these viruses. These viral supernatants are used to infect endothelial cells *in vitro* and iNOS activity assayed at 48-72 hours after infection as described below (see figure 9 and 10).

To determine effective infection, endothelial cell nitric oxide production will be determined by several methods and compared to endothelial cells infected with control viruses. Nitric oxide produced by the intact cells can be quantified by measuring the release of NO₂⁻ + NO₃⁻ into the culture medium. Figures 9 and 10 demonstrate successful transfer of iNOS function to endothelial cells using both MFG-iNOS and DFG-iNOS-Neo^R vectors as evidenced by increased NO₂⁻ production in comparison to uninfected and control virus infected endothelial cells. Enzyme activity within the cells can be measured in cytosolic preparations from cultured cells. iNOS can be distinguished from native cNOS by excluding activity in the membrane fraction, where 70-90% of native cNOS is located. The presence of iNOS mRNA will be detected by Northern blot analysis. Based on the human iNOS sequence, a set of human specific PCR primers for iNOS has been designed which do not amplify the endothelial cNOS mRNA. iNOS protein will be sought by Western blot analysis of cytosolic proteins and immunohistochemistry of intact cells (to localize sites of expression within the cell). Previously characterized human and murine iNOS antibodies, as well as a human cNOS antibody will be utilized in Western blot analysis and immunohistochemical techniques. The immunohistochemistry will also allow for an estimation of the efficiency of infection by calculating the percentage of positive staining endothelial cells. The stability of iNOS expression in the endothelial cells will be followed over time through subsequent cell passages. Nitric oxide-induced toxicity will be determined by cellular morphology as well as by ³H-thymidine uptake for DNA synthesis. *In vitro* toxicity encountered due to

excessive nitric oxide production can be controlled by adding inhibitors such as N^G-monomethyl-L-arginine (NMA), which competitively inhibits the iNOS enzyme but does not effect gene expression. A second technique for limiting any nitric oxide toxicity is the addition of hemoglobin to the cultures. Hemoglobin rapidly binds and deactivates nitric oxide.

Endothelial cell transfection with naked DNA (e.g., including but not limited to a non-viral vector such a plasmid DNA construct) will also be performed so as to promote transient as well as long term expression of the iNOS DNA sequence in the target cell. The iNOS-containing construct is prepared for endothelial cell transfection by forming a DNA-cationic liposome complex as described by Gao and Huang (1991, Biochem. Biophys. Res. Comm. 179: 280-285). The human hepatocyte iNOS cDNA sequence has been subcloned into the pCIS expression vector (Genentech), which utilizes a CMV promoter/enhancer, resulting in high iNOS activity in transient transfection experiments. In addition to the CMV enhancer/promoter sequence of pCIS, sequences downstream of the promoter enhancer fragment of the 5370 bp mammalian expression plasmid include, from 5' - 3', a CMV intron, a polylinker sequence for ligation of the DNA fragment of interest, an SV40 polyadenylation site, an SV40 origin of replication, a DHFR cDNA fragment and the β -lactamase cDNA, which imparts ampicillin resistance. As discussed elsewhere in the specification, any number of mammalian expression vectors may be utilized to deliver the iNOS sequence of interest to the target cell. The pCIS-iNOS DNA will be combined with lipofectamine (BRL) at a ratio of 1 μ g DNA / 10 nmole liposomes and slowly added to endothelial cells. The cells will be incubated for 5 hours in serum-free media, followed by washing and assay for iNOS activity 48 hours later. The lipofectamine reagent has demonstrated approximately a 10% transfection efficiency in cultured murine endothelial cells. As well as promoting transient and long-term expression of iNOS, liposome transfection of vector DNA comprising an iNOS DNA sequence also provides a system for assay of potential nitric oxide toxicity as discussed above.

Any of the above disclosed strategies for targeting endothelial cell populations may be applied to directed transfection or infection of vascular smooth muscle cells. As an example and not intended as a limitation, Figure 11 depicts results of a pCIS-iNOS/lipofectamine transfection targeting vascular smooth muscle cells. Significant

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nitrite production is detected for pCIS-iNOS transfected vascular smooth muscle cells in the absence, but not the presence, of N^G -monoethylarginine. Additionally, no nitrite production was detected upon transfection with a control plasmid (pSV-lacZ), and a plasmid-less control with or without the addition of liposomes. As discussed throughout the specification, this method of targeting endothelial and/or vascular smooth muscle cells is especially preferred for *in situ* transfection of target cells lining the arterial lumen.

An additional mode of delivering a DNA sequence encoding iNOS utilizes a hybrid liposome:adeno-associated (AAV) construct. The use of liposome transfection procedures allows the use of an AAV viral vector comprising an iNOS DNA sequence. This recombinant AAV construct is cotransfected into target endothelial cells with a plasmid containing the *rep* gene of AAV, providing transient expression of the *rep* protein to enhance stable integration of the recombinant AAV genome into the target cell genome. To distinguish the transfected iNOS from the small amounts of native iNOS that may be expressed by endothelial cells, the iNOS constructs will include a hemagglutinin epitope tag. The epitope tag will be inserted both 5' and 3', and tested for any effects on iNOS activity. An antibody to the hemagglutinin epitope will be used to identify transfected iNOS by methods known to one of ordinary skill in the art.

In one example, but by no means forwarded as a limitation, the CMV promoter-iNOS region of pCIS-iNOS will be ligated between the terminal repeats of AAV. The iNOS-AAV construct will be cotransfected into endothelial cells with a plasmid containing the *rep* gene of AAV and with lipofectamine (BRL). An assay for iNOS activity will be assayed 48-72 hours later.

Additionally, an AAV-iNOS construct may be utilized to infect target endothelial cells (for a review of using AAV genomes as gene therapy based vectors, see Muzyczka, 1992, Current Topics in Microbiology and Immunology 158: 97-129). In utilizing the recombinant virus alone, human cells containing a helper virus such as adenovirus or herpes virus are transfected with a recombinant AAV plasmid comprising a human hepatocyte iNOS DNA sequence and a non-packaging complementing AAV plasmid which supplies wild type AAV gene products in *trans*. The recombinant AAV virus (comprising the iNOS DNA sequence) is purified away from contaminating helper virus and is then utilized to infect target endothelial cells.

13.1.2. IN VIVO ARTERIAL CELL MANIPULATION

Yucatan minipigs will be anesthetized with pentobarbital and iliofemoral arteries exposed by sterile technique. A specially designed double balloon catheter (C.R. Bard, Inc.) will be inserted into the iliac artery, partially inflated, and passed to mechanically
5 denude the endothelium, as described in Nabel, et al. (1989, Science 244: 1342-1344). The catheter is repositioned within the denuded segment, proximal and distal balloons are inflated, followed by heparin irrigation and 10 minute instillation of dispase, which removes any remaining endothelial cells. Subsequently, *in vitro* iNOS-infected
10 endothelial cells (2×10^6) will be instilled for 30 minutes, followed by catheter removal, arterial side branch ligation, and wound closure.

As a control, the contralateral iliofemoral artery will be seeded with endothelial cells infected with a control virus. Segments of experimental and control arteries will be removed 2 or 6 weeks later. Immunohistochemistry and iNOS enzyme assay will confirm
15 expression *in vivo*. Tissue fixation and electron microscopy will be performed to characterize endothelial cell and vascular morphology. Minimal nitric oxide-mediated toxicity to the vasculature and systemically is expected *in vivo*. Excess nitric oxide which escapes the cells should be scavenged immediately by hemoglobin in circulating red blood cells in the vessel lumen. Endothelial cell function will be confirmed by uptake of
20 acetylated low density lipoprotein, the presence of von Willebrand's factor, and angiotensin converting enzyme activity.

The procedure described in the previous two paragraphs can be performed by substituting *in vitro* iNOS-infected vascular smooth muscle cells for endothelial cells.

14. EXAMPLE: NOS DIRECTED CANCER THERAPY

iNOS-directed cancer therapy may be exemplified by harvesting and selective
25 culture of a patients tumor infiltrating lymphocyte population, transduction by an iNOS containing viral or non-viral vector, followed by reintroduction of the iNOS-transduced cell to the patient. Peripheral blood lymphocytes are removed from the patient and TILs are selected in culture as described in Rosenberg, et al. (1992, Human Gene Therapy
30 3:57-73, herein incorporated by reference). The TILs will then be utilized as the target cell population for transduction with DFG-iNOS-Neo^R. Transduced TILs are selected in

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G418-supplemented medium and prepared for administration back to the patient by known techniques.

Another method of iNOS-directed therapy for treating cancer is direct delivery of MFG-iNOS to tumor site(s) in liposome capsules. The stability of liposomes, coupled with the impermeable nature of these vesicles makes them useful vehicles for the delivery of iNOS containing sequences, such as but not limited to pCIS-iNOS, to the tumor site. Site specific delivery of the liposome capsule to the tumor site is promoted by modification of the liposome membrane to exhibit a tumor specific antibody so as to promote liposome adhesion and fusion only to the tumor cell. Local delivery of a recombinant iNOS vector to the tumor site will result in increased local iNOS expression, increased nitric oxide production and hence, antitumor activity.

pCIS-iNOS liposomes will be formulated into a suitable pharmaceutical carrier for *in vivo* administration by injection or surgical implant.

15. EXAMPLE: NOS DIRECTED ANTIMICROBIAL TREATMENT

The treatment of microbial infections by increasing local iNOS expression will be exemplified through the treatment of a malarial infection. The recombinant plasmid vector pCIS-NOS will be delivered locally to the liver in liposome capsules. The liposome capsules will be modified to exhibit a liver specific surface ligand. An asialoprotein is a glycoprotein treated to remove sialic acid (i.e., neuraminic acid). The resulting asialoprotein specifically binds to the galactose receptor unique to hepatocytes (see Wall, et. al., 1980, Cell 21: 79-83). Therefore, encapsulating pCIS-iNOS within an asialoprotein-containing liposome will ensure delivery to and local expression of iNOS in hepatocytes only.

The pCIS-iNOS vector incorporated into liposomes will be formulated into a suitable pharmaceutical carrier for *in vivo* administration by any appropriate route including but not limited to injection, absorption through epithelial or mucocutaneous lining or by a sustained released implant, whether it be a cellular or tissue implant.

16. DEPOSIT OF MICROORGANISMS

The following microorganisms have been deposited by David A. Geller on behalf of the University of Pittsburgh of the Commonwealth System of Higher Education, Pittsburgh,

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Pennsylvania 15260, USA, on November 18, 1992, with and are available from the permanent collection of the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 202852-1776, USA:

ATCC 75358 - Human Hepatocyte Inducible Nitric Oxide Synthase cDNA in
5 pBluescript (pHINOS)

ATCC 69126 - Human Hepatocyte Inducible Nitric Oxide Synthase cDNA in
pBluescript transformed in *E. coli* SOLR bacteria (plasmid HINOS cDNA)

10 The American Type Culture Collection has performed viability tests on each of the
hereinbefore mentioned deposited microorganisms and has concluded on November 20,
1992, that each of the hereinbefore mentioned deposited microorganisms is viable and
capable of reproduction.

15 These deposits are available to the public upon the grant of a patent to the
assignee, the University of Pittsburgh of the Commonwealth System of Higher Education,
disclosing them. However, it should be understood that the availability of these deposits
does not constitute a license to practice this invention in derogation of patent rights
granted by governmental action.

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SEQUENCE LISTING

5 (1) GENERAL INFORMATION:

(i) APPLICANT: Univeristy of Pittsburgh of the Commonwealth
System of Higher Education

10

(ii) TITLE OF INVENTION: Inducible Nitric Oxide Synthase
Gene for Treatment of Disease

15

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:

20

(A) ADDRESSEE: Lewis F. Gould, Jr.
Eckert Seamans Cherin & Mellott

(B) STREET: 1700 Market Street, Suite 3232

(C) CITY: Philadelphia

(D) STATE: PA

25

(E) COUNTRY: USA

(F) ZIP: 19103

(v) COMPUTER READABLE FORM:

30

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

35

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

40

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Gould, Lewis F. Jr.

(B) REGISTRATION NUMBER: 25,057

45

(C) REFERENCE/DOCKET NUMBER: 119130-2

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(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (215) 575-6020

(B) TELEFAX: (215) 575-6015

(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 4145 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(A) Human Hepatocyte Inducible Nitric Oxide

Synthase cDNA Clone

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(F) TISSUE TYPE: Induced Human Hepatocyte RNA

(vii) IMMEDIATE SOURCE:

(A) LIBRARY: Lambda Zap II cDNA

(B) CLONE: pHINOS

(viii) POSITION IN GENOME:

(A) CHROMOSOME/SEGMENT: unknown

(B) MAP POSITION: unknown

(C) UNITS: unknown

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 207..3668

(C) IDENTIFICATION METHOD: Experiment

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTGCTTTAAA ATCTCTCGGC CACCTTTGAT GAGGGGACTG GGCAGTTCTA GACAGTCCCG 60
AAGTTCTCAA GGCACAGGTC TCTTCCTGGT TTGACTGTCC TTACCCCGGG GAGGCAGTGC 120

BNSDOCID: <WO 9600006A1_1>

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5	CGC	TAT	GCT	GGC	TAC	CAG	ATG	CCA	GAT	GGC	AGC	ATC	AGA	GGG	GAC	CCT	1049
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10	GCC	AAC	GTG	GAA	TTC	ACT	CAG	CTG	TGC	ATC	GAC	CTG	GGC	TGG	AAG	CCC	1097
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	Ala	Met	Glu	His	Pro	Lys	Tyr	Glu	Trp	Phe	Arg	Glu	Leu	Glu	Leu	Lys	
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				365					370					375			
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			380					385					390				
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		410				415					420					425	
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	Phe	Gln	Lys	Gln	Asn	Val	Thr	Ile	Met	Asp	His	His	Ser	Ala	Ala	Glu	
					430					435					440		
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				445					450					455			
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	Pro	Ala	Asp	Trp	Ile	Trp	Leu	Val	Pro	Pro	Met	Ser	Gly	Ser	Ile	Thr	
			460					465					470				
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	Pro	Val	Phe	His	Gln	Glu	Met	Leu	Asn	Tyr	Val		Ser	Pro	Phe	Tyr	
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80	CGG	AGA	CCC	AAG	AGA	AGA	GAG	ATT	CCA	TTG	AAA	GTC	TTG	GTC	AAA	GCT	1769
	Arg	Arg	Pro	Lys	Arg	Arg	Glu	Ile	Pro	Leu	Lys	Val	Leu	Val	Lys	Ala	
					510					515					520		

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	GTG	CTC	TTT	GCC	TGT	ATG	CTG	ATG	CGC	AAG	ACA	ATG	GCG	TCC	CGA	GTC	1817
	Val	Leu	Phe	Ala	Cys	Met	Leu	Met	Arg	Lys	Thr	Met	Ala	Ser	Arg	Val	
				525					530					535			
5	AGA	GTC	ACC	ATC	CTC	TTT	GCG	ACA	GAG	ACA	GGA	AAA	TCA	GAG	GCG	CTG	1865
	Arg	Val	Thr	Ile	Leu	Phe	Ala	Thr	Glu	Thr	Gly	Lys	Ser	Glu	Ala	Leu	
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	Ala	Trp	Asp	Leu	Gly	Ala	Phe	Ser	Cys	Ala	Phe	Asn	Pro	Lys	Val		
		555					560					565					
15	GTC	TGC	ATG	GAT	AAG	TAC	AGG	CTG	AGC	TGC	CTG	GAG	GAG	GAA	CGG	CTG	1961
	Val	Cys	Met	Asp	Lys	Tyr	Arg	Leu	Ser	Cys	Leu	Glu	Glu	Glu	Arg	Leu	
	570					575					580					585	
20	CTG	TTG	GTG	GTG	ACC	AGT	ACG	TTT	GGC	AAT	GGA	GAC	TGC	CCT	GGC	AAT	2009
	Leu	Leu	Val	Val	Ser	Thr	Phe	Gly	Asn	Gly	Asp	Cys	Pro	Gly	Asn		
				590					595						600		
25	GGA	GAG	AAA	CTG	AAG	AAA	TCG	CTC	TTC	ATG	CTG	AAA	GAG	CTC	AAC	AAC	2057
	Gly	Glu	Lys	Leu	Lys	Lys	Ser	Leu	Phe	Met	Leu	Lys	Glu	Leu	Asn	Asn	
				605					610					615			
30	AAA	TTC	AGG	TAC	GCT	GTG	TTT	GGC	CTC	GGC	TCC	AGC	ATG	TAC	CCT	CGG	2105
	Lys	Phe	Arg	Tyr	Ala	Val	Phe	Gly	Leu	Gly	Ser	Ser	Met	Tyr	Pro	Arg	
			620					625					630				
35	TTC	TGC	GCC	TTT	GCT	CAT	GAC	ATT	GAT	CAG	AAG	CTG	TCC	CAC	CTG	GGG	2153
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		635					640					645					
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	Glu	Thr	Phe	Asp	Val	Arg	Gly	Lys	Gln	His	Ile	Gln	Ile	Pro	Lys	Leu	
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	Asp	Ser	Gln	Pro	Leu	Asp	Leu	Ser	Lys	Ala	Leu	Ser	Ser	Met	His	Ala	
			715				720						725				
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70	CCG	ACA	TCC	AGC	CGT	GCC	ACC	ATC	CTG	GTG	GAA	CTC	TCC	TGT	GAG	GAT	2489
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				750						755						760	
75	GGC	CAA	GGC	CTG	AAC	TAC	CTG	CCG	GGG	GAG	CAC	CTT	GGG	GTT	TGC	CCA	2537
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				765					770					775			
80	GGC	AAC	CAG	CCG	GCC	CTG	GTC	CAA	GGC	ATC	CTG	GAG	CGA	GTG	GTG	GAT	2585
	Gly	Asn	Gln	Pro	Ala	Leu	Val	Gln	Gly	Ile	Leu	Glu	Arg	Val	Val	Asp	
			780					785					790				

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	GGC	CCC	ACA	CCC	CAC	CAG	ACA	GTG	CGC	CTG	GAG	GAC	CTG	GAT	GAG	AGT	2633
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	795						800					805					
5	GGC	AGC	TAC	TGG	GTC	AGT	GAC	AAG	AGG	CTG	CCC	CCC	TGC	TCA	CTC	AGC	2681
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	810					815					820					825	
10	CAG	GCC	CTC	ACC	TAC	TCC	CCG	GAC	ATC	ACC	ACA	CCC	CCA	ACC	CAG	CTG	2729
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	Arg	Leu	Glu	Ala	Leu	Cys	Gln	Pro	Ser	Glu	Tyr	Ser	Lys	Trp	Lys	Phe	
			860					865					870				
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	Thr	Asn	Ser	Pro	Thr	Phe	Leu	Glu	Val	Leu	Glu	Glu	Phe	Pro	Ser	Leu	
	875						880					885					
30	CGG	GTG	TCT	GCT	GGC	TTC	CTG	CTT	TCC	CAG	CTC	CCC	ATT	CTG	AAG	CCC	2921
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	955						960					965					
55	CCC	GAG	GAT	CCC	TCC	CAT	CCT	TGC	ATC	CTC	ATC	GGG	CCT	GGC	ACA	GGC	3161
	Pro	Glu	Asp	Pro	Ser	His	Pro	Cys	Ile	Leu	Ile	Gly	Pro	Gly	Thr	Gly	
	970					975					980					985	
60	ATC	GTG	CCC	TTC	CGC	AGT	TTC	TGG	CAG	CAA	CGG	CTC	CAT	GAC	TCC	CAG	3209
	Ile	Val	Pro	Phe	Arg	Ser	Phe	Trp	Gln	Gln	Arg	Leu	His	Asp	Ser	Gln	
					990					995					1000		
65	CAC	AAG	GGA	GTG	CGG	GGA	GGC	CGC	ATG	ACC	TTG	GTG	TTT	GGG	TGC	CGC	3257
	His	Lys	Gly	Val	Arg	Gly	Gly	Arg	Met	Thr	Leu	Val	Phe	Gly	Cys	Arg	
				1005					1010					1015			
70	CGC	CCA	GAT	GAG	GAC	CAC	ATC	TAC	CAG	GAG	GAG	ATG	CTG	GAG	ATG	GCC	3305
	Arg	Pro	Asp	Glu	Asp	His	Ile	Tyr	Gln	Glu	Glu	Met	Leu	Glu	Met	Ala	
			1020					1025					1030				
75	CAG	AAG	GGG	GTG	CTG	CAT	GCG	GTG	CAC	ACA	GCC	TAT	TCC	CGC	CTG	CCT	3353
	Gln	Lys	Gly	Val	Leu	His	Ala	Val	His	Thr	Ala	Tyr	Ser	Arg	Leu	Pro	
		1035					1040					1045					
80	GGC	AAG	CCC	AAG	GTC	TAT	GTT	CAG	GAC	ATC	CTG	CGG	CAG	CAG	CTG	GCC	3401
	Gly	Lys	Pro	Lys	Val	Tyr	Val	Gln	Asp	Ile	Leu	Arg	Gln	Gln	Leu	Ala	
	1050					1055					1060					1065	

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	AGC GAG GTG CTC CGT GTG CTC CAC AAG GAG CCA GGC CAC CTC TAT GTT	3449
	Ser Glu Val Leu Arg Val Leu His Lys Glu Pro Gly His Leu Tyr Val	
	1070 1075 1080	
5	TGC GGG GAT GTG CGC ATG GCC CGG GAC GTG GCC CAC ACC CTG AAG CAG	3497
	Cys Gly Asp Val Arg Met Ala Arg Asp Val Ala His Thr Leu Lys Gln	
	1085 1090 1095	
10	CTG GTG GCT GCC AAG CTG AAA TTG AAT GAG GAG CAG GTC GAG GAC TAT	3545
	Leu Val Ala Ala Lys Leu Lys Leu Asn Glu Glu Gln Val Glu Asp Tyr	
	1100 1105 1110	
15	TTC TTT CAG CTC AAG AGC CAG AAG CGC TAT CAC GAA GAT ATC TTC GGT	3593
	Phe Phe Gln Leu Lys Ser Gln Lys Arg Tyr His Glu Asp Ile Phe Gly	
	1115 1120 1125	
20	GCT GTA TTT CCT TAC GAG GCG AAG AAG GAC AGG GTG GCG GTG CAG CCC	3641
	Ala Val Phe Pro Tyr Glu Ala Lys Lys Asp Arg Val Ala Val Gln Pro	
	1130 1135 1140 1145	
	AGC AGC CTG GAG ATG TCA GCG CTC TGAGGGCCTA CAGGAGGGGT TAAAGCTGCC	3695
	Ser Ser Leu Glu Met Ser Ala Leu	
	1150	
25	GGCACAGAAC TTAAGGATGG AGCCAGCTCT GCATTATCTG AGGTCACAGG GCCTGGGGAG	3755
	ATGGAGGAAA GTGATATCCC CCAGCCTCAA GTCTTATTTC CTCAACGTTG CTCCCCATCA	3815
30	AGCCCTTTAC TTGACCTCCT AACAAGTAGC ACCCTGGATT GATCGGAGCC TCCTCTCTCA	3875
	AACTGGGGCC TCCCTGGTCC CTTGGAGACA AAATCTTAA TGCCAGGCCT GGCGAGTGGG	3935
	TGAAAGATGG AACTTGCTGC TGAGTGCACC ACTTCAAGTG ACCACCAGGA GGTGCTATCG	3995
35	CACCACTGTG TATTAACTG CCTTGTGTAC AGTTATTTAT GCCTCTGTAT TTAAAAAACT	4055
	AACACCCAGT CTGTTCCCCA TGGCCACTTG GGTCTTCCCT GTATGATTCC TTGATGGAGA	4115
40	TATTACATG AATTGCATTT TACTTTAATC	4145

(2) INFORMATION FOR SEQ ID NO:2:

45

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1153 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

50

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

55

Met Ala Cys Pro Trp Lys Phe Leu Phe Lys Thr Lys Phe His Gln Tyr
 1 5 10 15

60

Ala Met Asn Gly Glu Lys Asp Ile Asn Asn Asn Val Glu Lys Ala Pro
 20 25 30

Cys Ala Thr Ser Ser Pro Val Thr Gln Asp Asp Leu Gln Tyr His Asn
 35 40 45

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Leu Ser Lys Gln Gln Asn Glu Ser Pro Gln Pro Leu Val Glu Thr Gly
 50 55 60
 5 Lys Lys Ser Pro Glu Ser Leu Val Lys Leu Asp Ala Thr Pro Leu Ser
 65 70 75 80
 Ser Pro Arg His Val Arg Ile Lys Asn Trp Gly Ser Gly Met Thr Phe
 85 90 95
 10 Gln Asp Thr Leu His His Lys Ala Lys Gly Ile Leu Thr Cys Arg Ser
 100 105 110
 Lys Ser Cys Leu Gly Ser Ile Met Thr Pro Lys Ser Leu Thr Arg Gly
 115 120 125
 15 Pro Arg Asp Lys Pro Thr Pro Pro Asp Glu Leu Leu Pro Gln Ala Ile
 130 135 140
 20 Glu Phe Val Asn Gln Tyr Tyr Gly Ser Phe Lys Glu Ala Lys Ile Glu
 145 150 155 160
 Glu His Leu Ala Arg Val Glu Ala Val Thr Lys Glu Ile Glu Thr Thr
 165 170 175
 25 Gly Thr Tyr Gln Leu Thr Gly Asp Glu Leu Ile Phe Ala Thr Lys Gln
 180 185 190
 Ala Trp Arg Asn Ala Pro Arg Cys Ile Gly Arg Ile Gln Trp Ser Asn
 195 200 205
 30 Leu Gln Val Phe Asp Ala Arg Ser Cys Ser Thr Ala Arg Glu Met Phe
 210 215 220
 35 Glu His Ile Cys Arg His Val Arg Tyr Ser Thr Asn Asn Gly Asn Ile
 225 230 235 240
 Arg Ser Ala Ile Thr Val Phe Pro Gln Arg Ser Asp Gly Lys His Asp
 245 250 255
 40 Phe Arg Val Trp Asn Ala Gln Leu Ile Arg Tyr Ala Gly Tyr Gln Met
 260 265 270
 Pro Asp Gly Ser Ile Arg Gly Asp Pro Ala Asn Val Glu Phe Thr Gln
 275 280 285
 45 Leu Cys Ile Asp Leu Gly Trp Lys Pro Lys Tyr Gly Arg Phe Asp Val
 290 295 300
 50 Val Pro Leu Val Leu Gln Ala Asn Gly Arg Asp Pro Glu Leu Phe Glu
 305 310 315 320
 Ile Pro Pro Asp Leu Val Leu Glu Val Ala Met Glu His Pro Lys Tyr
 325 330 335
 55 Glu Trp Phe Arg Glu Leu Glu Leu Lys Trp Tyr Ala Leu Pro Ala Val
 340 345 350
 Ala Asn Met Leu Leu Glu Val Gly Gly Leu Glu Phe Pro Gly Cys Pro
 355 360 365
 60 Phe Asn Gly Trp Tyr Met Gly Thr Glu Ile Gly Val Arg Asp Phe Cys
 370 375 380
 65 Asp Val Gln Arg Tyr Asn Ile Leu Glu Glu Val Gly Arg Arg Met Gly
 385 390 395 400

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	Leu	Glu	Thr	His	Lys	Leu	Ala	Ser	Leu	Trp	Lys	Asp	Gln	Ala	Val	Val
					405					410					415	
5	Glu	Ile	Asn	Ile	Ala	Val	Ile	His	Ser	Phe	Gln	Lys	Gln	Asn	Val	Thr
				420					425					430		
	Ile	Met	Asp	His	His	Ser	Ala	Ala	Glu	Ser	Phe	Met	Lys	Tyr	Met	Gln
			435					440					445			
10	Asn	Glu	Tyr	Arg	Ser	Arg	Gly	Gly	Cys	Pro	Ala	Asp	Trp	Ile	Trp	Leu
		450					455					460				
	Val	Pro	Pro	Met	Ser	Gly	Ser	Ile	Thr	Pro	Val	Phe	His	Gln	Glu	Met
	465					470					475					480
15	Leu	Asn	Tyr	Val	Leu	Ser	Pro	Phe	Tyr	Tyr	Tyr	Gln	Val	Glu	Ala	Trp
					485				490						495	
	Lys	Thr	His	Val	Trp	Gln	Asp	Glu	Lys	Arg	Arg	Pro	Lys	Arg	Arg	Glu
20				500					505					510		
	Ile	Pro	Leu	Lys	Val	Leu	Val	Lys	Ala	Val	Leu	Phe	Ala	Cys	Met	Leu
			515					520					525			
25	Met	Arg	Lys	Thr	Met	Ala	Ser	Arg	Val	Arg	Val	Thr	Ile	Leu	Phe	Ala
		530					535					540				
	Thr	Glu	Thr	Gly	Lys	Ser	Glu	Ala	Leu	Ala	Trp	Asp	Leu	Gly	Ala	Leu
30		545				550					555					560
	Phe	Ser	Cys	Ala	Phe	Asn	Pro	Lys	Val	Val	Cys	Met	Asp	Lys	Tyr	Arg
					565					570					575	
35	Leu	Ser	Cys	Leu	Glu	Glu	Glu	Arg	Leu	Leu	Leu	Val	Val	Thr	Ser	Thr
				580					585					590		
	Phe	Gly	Asn	Gly	Asp	Cys	Pro	Gly	Asn	Gly	Glu	Lys	Leu	Lys	Lys	Ser
			595					600					605			
40	Leu	Phe	Met	Leu	Lys	Glu	Leu	Asn	Asn	Lys	Phe	Arg	Tyr	Ala	Val	Phe
		610					615					620				
	Gly	Leu	Gly	Ser	Ser	Met	Tyr	Pro	Arg	Phe	Cys	Ala	Phe	Ala	His	Asp
45		625				630					635					640
	Ile	Asp	Gln	Lys	Leu	Ser	His	Leu	Gly	Ala	Ser	Gln	Leu	Thr	Pro	Met
					645				650						655	
50	Gly	Glu	Gly	Asp	Glu	Leu	Ser	Gly	Gln	Glu	Asp	Ala	Phe	Arg	Ser	Trp
				660					665					670		
	Ala	Val	Gln	Thr	Phe	Lys	Ala	Ala	Cys	Glu	Thr	Phe	Asp	Val	Arg	Gly
			675					680					685			
55	Lys	Gln	His	Ile	Gln	Ile	Pro	Lys	Leu	Tyr	Thr	Ser	Asn	Val	Thr	Trp
							695					700				
	Asp	Pro	His	His	Tyr	Arg	Leu	Val	Gln	Asp	Ser	Gln	Pro	Leu	Asp	Leu
60		705				710					715					720
	Ser	Lys	Ala	Leu	Ser	Ser	Met	His	Ala	Lys	Asn	Val	Phe	Thr	Met	Arg
					725					730					735	
65	Leu	Lys	Ser	Arg	Gln	Asn	Leu	Gln	Ser	Pro	Thr	Ser	Ser	Arg	Ala	Thr
				740					745					750		

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Ile Leu Val Glu Leu Ser Cys Glu Asp Gly Gln Gly Leu Asn Tyr Leu
 755 760 765
 5 Pro Gly Glu His Leu Gly Val Cys Pro Gly Asn Gln Pro Ala Leu Val
 770 775 780
 Gln Gly Ile Leu Glu Arg Val Val Asp Gly Pro Thr Pro His Gln Thr
 785 790 795 800
 10 Val Arg Leu Glu Asp Leu Asp Glu Ser Gly Ser Tyr Trp Val Ser Asp
 805 810 815
 Lys Arg Leu Pro Pro Cys Ser Leu Ser Gln Ala Leu Thr Tyr Ser Pro
 820 825 830
 15 Asp Ile Thr Thr Pro Pro Thr Gln Leu Leu Leu Gln Lys Leu Ala Gln
 835 840 845
 Val Ala Thr Glu Glu Pro Glu Arg Gln Arg Leu Glu Ala Leu Cys Gln
 850 855 860
 20 Pro Ser Glu Tyr Ser Lys Trp Lys Phe Thr Asn Ser Pro Thr Phe Leu
 865 870 875 880
 25 Glu Val Leu Glu Glu Phe Pro Ser Leu Arg Val Ser Ala Gly Phe Leu
 885 890 895
 Leu Ser Gln Leu Pro Ile Leu Lys Pro Arg Phe Tyr Ser Ile Ser Ser
 900 905 910
 30 Ser Arg Asp His Thr Pro Thr Glu Ile His Leu Thr Val Ala Val Val
 915 920 925
 Thr Tyr His Thr Gly Asp Gly Gln Gly Pro Leu His His Gly Val Cys
 930 935 940
 35 Ser Thr Trp Leu Asn Ser Leu Lys Pro Gln Asp Pro Val Pro Cys Phe
 945 950 955 960
 40 Val Arg Asn Ala Ser Ala Phe His Leu Pro Glu Asp Pro Ser His Pro
 965 970 975
 Cys Ile Leu Ile Gly Pro Gly Thr Gly Ile Val Pro Phe Arg Ser Phe
 980 985 990
 45 Trp Gln Gln Arg Leu His Asp Ser Gln His Lys Gly Val Arg Gly Gly
 995 1000 1005
 50 Arg Met Thr Leu Val Phe Gly Cys Arg Arg Pro Asp Glu Asp His Ile
 1010 1015 1020
 Tyr Gln Glu Glu Met Leu Glu Met Ala Gln Lys Gly Val Leu His Ala
 1025 1030 1035 1040
 55 Val His Thr Ala Tyr Ser Arg Leu Pro Gly Lys Pro Lys Val Tyr Val
 1045 1050 1055
 Gln Asp Ile Leu Arg Gln Gln Leu Ala Ser Glu Val Leu Arg Val Leu
 1060 1065 1070
 60 His Lys Glu Pro Gly His Leu Tyr Val Cys Gly Asp Val Arg Met Ala
 1075 1080 1085
 65 Arg Asp Val Ala His Thr Leu Lys Gln Leu Val Ala Ala Lys Leu Lys
 1090 1095 1100

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Leu Asn Glu Glu Gln Val Glu Asp Tyr Phe Phe Gln Leu Lys Ser Gln
1105 1110 1115 1120

5 Lys Arg Tyr His Glu Asp Ile Phe Gly Ala Val Phe Pro Tyr Glu Ala
1125 1130 1135

Lys Lys Asp Arg Val Ala Val Gln Pro Ser Ser Leu Glu Met Ser Ala
1140 1145 1150

10 Leu

WHAT IS CLAIMED IS:

1. A method of treating a disease or disorder within a mammalian host which comprises transferring an isolated nucleic acid fragment to a population of mammalian cells, said nucleic acid fragment encoding a biologically active iNOS protein or protein fragment such that local increases in iNOS expression impart therapeutic relief from said disease or disorder.
2. The method of claim 1 wherein said mammalian host is a human.
3. The method of claim 2 wherein said disease or disorder is a vascular disease or disorder.
4. The method of claim 3 wherein said isolated nucleic acid fragment encoding a biologically active iNOS protein or iNOS protein fragment is an isolated human DNA sequence.
5. The method of claim 4 wherein said population of mammalian cells are selected from the group consisting of endothelial cells, vascular smooth muscle cells and a combination of endothelial cells and vascular smooth muscle cells.
6. The method of claim 5, which comprises:
 - (a) culturing said mammalian cells *in vitro*;
 - (b) incubating said mammalian cells of step (a) with said human DNA sequence encoding a biologically active iNOS protein or iNOS protein fragment, resulting in iNOS-transfected mammalian cells;
 - (c) substantially clearing an occluded region of an artery;
 - (d) populating said artery with said iNOS-transfected mammalian cells so as to promote luminal attachment and subsequent expression of said human DNA sequence, resulting in local increases in iNOS concentration.
7. The method of claim 6 wherein step (c) is accomplished by an endovascular surgical procedure selected from the group consisting of balloon angioplasty, laser-assisted balloon angioplasty, mechanical endarterectomy and vascular endoscopy.
8. The method of claim 7 wherein step (d) involves utilizing a double balloon catheter to physically segregate the region of said artery and for delivering said iNOS-transfected mammalian cells.

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9. The method of claim 7 wherein said human DNA sequence encoding a biologically active iNOS protein or iNOS protein fragment is presented to said cultured mammalian cells as a recombinant viral vector.

10. The method of claim 8 wherein said human DNA sequence encoding a biologically active iNOS protein or iNOS protein fragment is presented to said cultured mammalian cells as a recombinant retroviral vector.

11. The method of claim 9 wherein said recombinant retroviral vector is a recombinant MoMLV retroviral vector.

12. The method of claim 10 wherein said recombinant retroviral vector is a recombinant MoMLV retroviral vector.

13. The method of claim 11 wherein said recombinant MoMLV vector is MFG-iNOS.

14. The method of claim 11 wherein said recombinant MoMLV vector is DFG-iNOS-Neo.

15. The method of claim 12 wherein said recombinant MoMLV vector is MFG-iNOS.

16. The method of claim 12 wherein said recombinant MoMLV vector is DFG-iNOS-Neo.

17. The method of claim 8 wherein said human DNA sequence is presented to said cultured mammalian cells as a recombinant plasmid DNA vector by liposome-mediated transfection.

18. The method of claim 17 wherein said recombinant plasmid DNA vector is pCIS-iNOS.

19. The method of claim 8 wherein said recombinant viral vector is an adenovirus vector.

20. The method of claim 8 wherein said recombinant viral vector is an adeno-associated vector.

21. A method of treating a human vascular disease or disorder characterized by at least partial occlusion of an artery which comprises:

- (a) substantially clearing an occluded region of said arterial vessel;
- (b) physically segregating the cleared region of said arterial vessel;
- (c) delivering a population of nucleic acid fragments encoding a biologically active iNOS protein or protein fragment to the cleared region of said artery

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vessel so as to promote *in situ* transfection of mammalian cells selected from the group of arterial luminal cells consisting of endothelial cells, vascular smooth muscle cells and a combination of endothelial cells and vascular smooth muscle cells such that expression of iNOS within said *in situ* transfected mammalian cells impart therapeutic relief from said human vascular disease or disorder.

22. The method of claim 21 wherein step (a) is accomplished by endovascular surgical procedures selected from the group consisting of balloon angioplasty, laser-assisted balloon angioplasty, mechanical endarterectomy and vascular endoscopy.

23. The method of claim 22 wherein step (b) and step (c) involves utilizing a double balloon catheter to physically segregate the cleared region of said artery.

24. The method of claim 23 wherein said nucleic acid fragment encoding a biologically active iNOS protein or iNOS protein fragment is presented to said *in situ* mammalian cells as a recombinant viral vector.

25. The method of claim 24 wherein said recombinant retroviral vector is a recombinant MoMLV retroviral vector.

26. The method of claim 25 wherein said recombinant MoMLV vector is MFG-iNOS.

27. The method of claim 25 wherein said recombinant MoMLV vector is DFG-iNOS-Neo.

28. The method of claim 21 wherein said nucleic acid fragment is presented to said *in situ* mammalian cells as a recombinant plasmid DNA vector by liposome-mediated transfection.

29. The method of claim 28 wherein said recombinant plasmid DNA vector is pCIS-iNOS.

30. The method of claim 21 wherein said recombinant viral vector is an adenovirus vector.

31. The method of claim 21 wherein said recombinant viral vector is an adeno-associated virus vector.

32. A method of treating a patient with a vascular disease or disorder through vascular bypass surgery which comprises:

(a) transfecting *in vitro* cultured mammalian cells selected from the group consisting of endothelial cells, vascular smooth muscle cells and a combination of

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endothelial cells and vascular smooth muscle cells with a population of isolated nucleic acid fragments encoding a biologically active iNOS protein or protein fragment, resulting in a population of iNOS-transfected mammalian cells;

(b) seeding a synthetic or autogenous conduit with said population of iNOS-transfected mammalian cells;

(c) forming a proximal and a distal anastomosis to bypass an artery within said patient, so as to promote expression of iNOS within said iNOS-transfected mammalian cells associated with said synthetic or autogenous conduit, resulting in prophylactic and therapeutic relief from said vascular disease or disorder.

33. The method of claim 32 wherein said nucleic acid fragment encoding a biologically active iNOS protein or iNOS protein fragment is presented to said cultured mammalian cells as a recombinant viral vector.

34. The method of claim 33 wherein said recombinant retroviral vector is a recombinant MoMLV retroviral vector.

35. The method of claim 34 wherein said recombinant MoMLV vector is MFG-iNOS.

36. The method of claim 34 wherein said recombinant MoMLV vector is DFG-iNOS-Neo.

37. The method of claim 32 wherein said nucleic acid fragment is presented to said cultured mammalian cells as a recombinant plasmid DNA vector by liposome-mediated transformation.

38. The method of claim 37 wherein said recombinant DNA vector is pCIS-iNOS.

39. The method of claim 32 wherein said recombinant viral vector is an adenovirus vector.

40. The method of claim 32 wherein said recombinant viral vector is an adeno-associated virus vector.

41. The method of claim 32 wherein said synthetic bypass graft is a graft composed of polytetrafluoroethylene.

42. The method of claim 41 wherein said nucleic acid fragment encoding a biologically active iNOS protein or iNOS protein fragment is presented to said cultured mammalian cells as a recombinant viral vector.

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43. The method of claim 42 wherein said recombinant retroviral vector is a recombinant MoMLV retroviral vector.

44. The method of claim 43 wherein said recombinant MoMLV vector is MFG-iNOS.

45. The method of claim 43 wherein said recombinant MoMLV vector is DFG-iNOS-Neo.

46. The method of claim 41 wherein said nucleic acid fragment is presented to said cultured endothelial cells as a recombinant plasmid DNA vector by liposome-mediated transformation.

47. The method of claim 46 wherein said recombinant DNA vector is pCIS-iNOS.

48. The method of claim 41 wherein said recombinant viral vector is an adenovirus vector.

49. The method of claim 41 wherein said recombinant viral vector is an adeno-associated virus vector.

50. A method of treating a patient with a vascular disease or disorder through vascular bypass surgery which comprises:

(a) transfecting *in vitro* cultured mammalian cells selected from the group consisting of endothelial cells, vascular smooth muscle cells and a combination of endothelial cells and vascular smooth muscle cells with a population of isolated nucleic acid fragments encoding a biologically active iNOS protein or protein fragment, resulting in a population of iNOS-transfected mammalian cells;

(b) forming a proximal and a distal anastomosis to bypass a diseased portion of an artery within said patient;

(c) physically segregating said anastomoses subsequent to graft suturing;

(d) seeding the isolated areas of said anastomoses with said iNOS-transfected mammalian cells so as to repopulate the arterial wall of said artery within proximity of said anastomoses such that increases in local iNOS concentration impart prophylactic and therapeutic relief from said disease or disorder.

51. The method of claim 50 wherein said anastomosis of step (c) is said distal anastomosis.

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52. The method of claim 51 wherein step (c) involves utilizing a double balloon catheter to physically isolate the manipulated segment of said artery.

53. The method of claim 52 wherein said nucleic acid fragment encoding a biologically active iNOS protein or iNOS protein fragment is presented to said *in situ* mammalian cells as a recombinant viral vector.

54. The method of claim 53 wherein said recombinant retroviral vector is a recombinant MoMLV retroviral vector.

55. The method of claim 54 wherein said recombinant MoMLV vector is MFG-iNOS.

56. The method of claim 54 wherein said recombinant MoMLV vector is DFG-iNOS-Neo.

57. The method of claim 52 wherein said nucleic acid fragment is presented to said *in situ* mammalian cells as a recombinant plasmid DNA vector by liposome-mediated transfection.

58. The method of claim 57 wherein said recombinant DNA vector is pCIS-iNOS.

59. The method of claim 52 wherein said recombinant viral vector is an adenovirus vector.

60. The method of claim 52 wherein said recombinant viral vector is an adeno-associated virus vector.

61. A method of treating a patient with a vascular disease or disorder through vascular bypass surgery which comprises:

(a) forming a proximal and a distal anastomosis to bypass an artery within said patient;

(b) physically isolating said anastomoses subsequent to graft suturing; and,

(c) transfecting *in situ* mammalian cells lining the lumen at or around said anastomosis with an isolated nucleic acid fragment encoding a biologically active iNOS protein or protein fragment, said mammalian cells selected from the group of arterial luminal cells consisting of endothelial cells, vascular smooth muscle cells and a combination of endothelial cells and vascular smooth muscle cells, such that expression of

iNOS within said *in situ* mammalian cells imparts prophylactic and therapeutic relief from said human vascular disease or disorder.

62. The method of claim 61 wherein said anastomosis of step (b) is said distal anastomosis.

63. The method of claim 62 wherein step (a) involves utilizing a double balloon catheter to physically isolate the cleared region of said artery.

64. The method of claim 63 wherein said nucleic acid fragment encoding a biologically active iNOS protein or iNOS protein fragment is presented to said *in situ* mammalian cells as a recombinant viral vector.

65. The method of claim 64 wherein said recombinant retroviral vector is a recombinant MoMLV retroviral vector.

66. The method of claim 65 wherein said recombinant MoMLV vector is MFG-iNOS.

67. The method of claim 65 wherein said recombinant MoMLV vector is DFG-iNOS-Neo.

68. The method of claim 63 wherein said nucleic acid fragment is presented to said *in situ* mammalian cells as a recombinant plasmid DNA vector by liposome-mediated transfection.

69. The method of claim 68 wherein said recombinant DNA vector is pCIS-iNOS.

70. The method of claim 63 wherein said recombinant viral vector is an adenovirus vector.

71. The method of claim 63 wherein said recombinant viral vector is an adeno-associated virus vector.

72. A retroviral mammalian expression vector comprising an isolated iNOS DNA fragment of SEQ ID NO:1 or a biologically active fragment thereof.

73. A retroviral mammalian expression vector of claim 72 which is an MFG-based vector.

74. The MFG-based retroviral mammalian expression vector of claim 73 which is MFG-iNOS, as depicted in Figure 7.

75. The MFG-based retroviral mammalian expression vector of claim 73 which is DFG-iNOS-Neo, as depicted in Figure 8.

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76. A DNA plasmid expression vector comprising an isolated iNOS DNA fragment of SEQ ID NO:1 or a biologically active fragment thereof.

77. The DNA plasmid expression vector of claim 76 which is pCIS-iNOS.

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CTGCTTTAAA ATCTCTCGGC CACCTTTGAT GAGGGGACTG GGCAGTTCTA GACAGTCCCG	60
AAGTTCTCAA GGCACAGGTC TCTTCCTGGT TTGACTGTCC TTACCCCGGG GAGGCAGTGC	120
AGCCAGCTGC AAGCCCCACA GTGAAGAACA TCTGAGCTCA AATCCAGATA AGTGACATAA	180
GTGACCTGCT TTGTAAAGCC ATAGAG ATG GCC TGT CCT TGG AAA TTT CTG TTC	233
Met ₁ Ala Cys Pro Trp ₅ Lys Phe Leu Phe	
AAG ACC AAA TTC CAC CAG TAT GCA ATG AAT GGG GAA AAA GAC ATC AAC	281
Lys ₁₀ Thr Lys Phe His ₁₅ Gln Tyr Ala Met Asn Gly ₂₀ Glu Lys Asp Ile Asn ₂₅	
AAC AAT GTG GAG AAA GCC CCC TGT GCC ACC TCC AGT CCA GTG ACA CAG	329
Asn Asn Val Glu Lys ₃₀ Ala Pro Cys Ala Thr ₃₅ Ser Ser Pro Val Thr ₄₀ Gln	
GAT GAC CTT CAG TAT CAC AAC CTC AGC AAG CAG CAG AAT GAG TCC CCG	377
Asp Asp Leu Gln ₄₅ Tyr His Asn Leu Ser ₅₀ Lys Gln Gln Asn Glu ₅₅ Ser Pro	
CAG CCC CTC GTG GAG ACG GGA AAG AAG TCT CCA GAA TCT CTG GTC AAG	425
Gln Pro Leu ₆₀ Val Glu Thr Gly Lys ₆₅ Lys Ser Pro Glu Ser ₇₀ Leu Val Lys	
CTG GAT GCA ACC CCA TTG TCC TCC CCA CGG CAT GTG AGG ATC AAA AAC	473
Leu Asp ₇₅ Ala Thr Pro Leu Ser ₈₀ Ser Pro Arg His Val ₈₅ Arg Ile Lys Asn	
TGG GGC AGC GGG ATG ACT TTC CAA GAC ACA CTT CAC CAT AAG GCC AAA	521
Trp ₉₀ Gly Ser Gly Met Thr ₉₅ Phe Gln Asp Thr Leu ₁₀₀ His His Lys Ala Lys ₁₀₅	
GGG ATT TTA ACT TGC AGG TCC AAA TCT TGC CTG GGG TCC ATT ATG ACT	569
Gly Ile Leu Thr Cys ₁₁₀ Arg Ser Lys Ser Cys ₁₁₅ Leu Gly Ser Ile Met ₁₂₀ Thr	
CCC AAA AGT TTG ACC AGA GGA CCC AGG GAC AAG CCT ACC CCT CCA GAT	617
Pro Lys Ser Leu ₁₂₅ Thr Arg Gly Pro Arg ₁₃₀ Asp Lys Pro Thr Pro ₁₃₅ Pro Asp	

FIG. 1A

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GAG Glu	CTT Leu	CTA Leu 140	CCT Pro	CAA Gln	GCT Ala	ATC Ile	GAA Glu 145	TTT Phe	GTC Val	AAC Asn	CAA Gln	TAT Tyr 150	TAC Tyr	GGC Gly	TCC Ser	665
TTC Phe	AAA Lys 155	GAG Glu	GCA Ala	AAA Lys	ATA Ile	GAG Glu 160	GAA Glu	CAT His	CTG Leu	GCC Ala	AGG Arg 165	GTG Val	GAA Glu	GCG Ala	GTA Val	713
ACA Thr 170	AAG Lys	GAG Glu	ATA Ile	GAA Glu	ACA Thr 175	ACA Thr	GGA Gly	ACC Thr	TAC Tyr	CAA Gln 180	CTG Leu	ACG Thr	GGA Gly	GAT Asp	GAG Glu 185	761
CTC Leu	ATC Ile	TTC Phe	GCC Ala	ACC Thr 190	AAG Lys	CAG Gln	GCC Ala	TGG Trp	CGC Arg 195	AAT Asn	GCC Ala	CCA Pro	CGC Arg	TGC Cys 200	ATT Ile	809
GGG Gly	AGG Arg	ATC Ile	CAG Gln 205	TGG Trp	TCC Ser	AAC Asn	CTG Leu	CAG Gln 210	GTC Val	TTC Phe	GAT Asp	GCC Ala	CGC Arg 215	AGC Ser	TGT Cys	857
TCC Ser	ACT Thr	GCC Ala 220	CGG Arg	GAA Glu	ATG Met	TTT Phe	GAA Glu 225	CAC His	ATC Ile	TGC Cys	AGA Arg	CAC His 230	GTG Val	CGT Arg	TAC Tyr	905
TCC Ser	ACC Thr 235	AAC Asn	AAT Asn	GGC Gly	AAC Asn	ATC Ile 240	AGG Arg	TCG Ser	GCC Ala	ATC Ile	ACC Thr 245	GTG Val	TTC Phe	CCC Pro	CAG Gln	953
CGG Arg 250	AGT Ser	GAT Asp	GGC Gly	AAG Lys	CAC His 255	GAC Asp	TTC Phe	CGG Arg	GTG Val	TGG Trp 260	AAT Asn	GCT Ala	CAG Gln	CTC Leu	ATC Ile 265	1001
CGC Arg	TAT Tyr	GCT Ala	GGC Gly	TAC Tyr 270	CAG Gln	ATG Met	CCA Pro	GAT Asp	GGC Gly 275	AGC Ser	ATC Ile	AGA Arg	GGG Gly	GAC Asp 280	CCT Pro	1049
GCC Ala	AAC Asn	GTG Val	GAA Glu 285	TTC Phe	ACT Thr	CAG Gln	CTG Leu	TGC Cys 290	ATC Ile	GAC Asp	CTG Leu	GGC Gly	TGG Trp 295	AAG Lys	CCC Pro	1097
AAG Lys	TAC Tyr	GGC Gly 300	CGC Arg	TTC Phe	GAT Asp	GTG Val	GTC Val 305	CCC Pro	CTG Leu	GTC Val	CTG Leu	CAG Gln 310	GCC Ala	AAT Asn	GGC Gly	1145

FIG. 1B

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CGT Arg	GAC Asp 315	CCT Pro	GAG Glu	CTC Leu	TTC Phe	GAA Glu 320	ATC Ile	CCA Pro	CCT Pro	GAC Asp	CTT Leu 325	GTG Val	CTT Leu	GAG Glu	GTG Val	1193
GCC Ala 330	ATG Met	GAA Glu	CAT His	CCC Pro	AAA Lys 335	TAC Tyr	GAG Glu	TGG Trp	TTT Phe	CGG Arg 340	GAA Glu	CTG Leu	GAG Glu	CTA Leu	AAG Lys 345	1241
TGG Trp	TAC Tyr	GCC Ala	CTG Leu	CCT Pro 350	GCA Ala	GTG Val	GCC Ala	AAC Asn	ATG Met 355	CTG Leu	CTT Leu	GAG Glu	GTG Val	GGC Gly 360	GGC Gly	1289
CTG Leu	GAG Glu	TTC Phe	CCA Pro 365	GGG Gly	TGC Cys	CCC Pro	TTD Phe	AAT Asn 370	GGC Gly	TGG Trp	TAC Tyr	ATG Met	GGC Gly 375	ACA Thr	GAG Glu	1337
ATC Ile	GGA Gly	GTC Val 380	CGG Arg	GAC Asp	TTC Phe	TGT Cys	GAC Asp 385	GTC Val	CAG Gln	CGC Arg	TAC Tyr	AAC Asn 390	ATC Ile	CTG Leu	GAG Glu	1385
GAA Glu	GTG Val 395	GGC Gly	AGG Arg	AGA Arg	ATG Met	GGC Gly 400	CTG Leu	GAA Glu	ACG Thr	CAC His	AAG Lys 405	CTG Leu	GCC Ala	TCG Ser	CTC Leu	1433
TGG Trp 410	AAA Lys	GAC Asp	CAG Gln	GCT Ala	GTC Val 415	GTT Val	GAG Glu	ATC Ile	AAC Asn	ATT Ile 420	GCT Ala	GTG Val	ATC Ile	CAT His	AGT Ser 425	1481
TTT Phe	CAG Gln	AAG Lys	CAG Gln	AAT Asn 430	GTG Val	ACC Thr	ATC Ile	ATG Met	GAC Asp 435	CAC His	CAC His	TCG Ser	GCT Ala	GCA Ala 440	GAA Glu	1529
TCC Ser	TTC Phe	ATG Met	AAG Lys 445	TAC Tyr	ATG Met	CAG Gln	AAT Asn	GAA Glu 450	TAC Tyr	CGG Arg	TCC Ser	CGT Arg	GGG Gly 455	GGC Gly	TGC Cys	1577
CCG Pro	GCA Ala	GAC Asp 460	TGG Trp	ATT Ile	TGG Trp	CTG Leu	GTC Val 465	CCT Pro	CCC Pro	ATG Met	TCT Ser	GGG Gly 470	AGC Ser	ATC Ile	ACC Thr	1625
CCC Pro	GTG Val 475	TTT Phe	CAC His	CAG Gln	GAG Glu	ATG Met 480	CTG Leu	AAC Asn	TAC Tyr	GTC Val	CTG Leu 485	TCC Ser	CCT Pro	TTC Phe	TAC Tyr	1673

FIG. 1C

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TAC Tyr 490	TAT Tyr	CAG Gln	GTA Val	GAG Glu	GCC Ala 495	TGG Trp	AAA Lys	ACC Thr	CAT His	GTC Val 500	TGG Trp	CAG Gln	GAC Asp	GAG Glu	AAG Lys 505	1721
CGG Arg	AGA Arg	CCC Pro	AAG Lys	AGA Arg 510	AGA Arg	GAG Glu	ATT Ile	CCA Pro	TTG Leu 515	AAA Lys	GTC Val	TTG Leu	GTC Val	AAA Lys 520	GCT Ala	1769
GTG Val	CTC Leu	TTT Phe	GCC Ala 525	TGT Cys	ATG Met	CTG Leu	ATG Met	CGC Arg 530	AAG Lys	ACA Thr	ATG Met	GCG Ala	TCC Ser 535	CGA Arg	GTC Val	1817
AGA Arg	GTC Val	ACC Thr 540	ATC Ile	CTC Leu	TTT Phe	GCG Ala	ACA Thr 545	GAG Glu	ACA Thr	GGA Gly	AAA Lys	TCA Ser 550	GAG Glu	GCG Ala	CTG Leu	1865
GCC Ala	TGG Trp 555	GAC Asp	CTG Leu	GGG Gly	GCC Ala	TTA Leu 560	TTC Phe	AGC Ser	TGT Cys	GCC Ala	TTC Phe 565	AAC Asn	CCC Pro	AAG Lys	GTT Val	1913
GTC Val 570	TGC Cys	ATG Met	GAT Asp	AAG Lys	TAC Tyr 575	AGG Arg	CTG Leu	AGC Ser	TGC Cys	CTG Leu 580	GAG Glu	GAG Glu	GAA Glu	CGG Arg	CTG Leu 585	1961
CTG Leu	TTG Leu	GTG Val	GTG Val	ACC Thr 590	AGT Ser	ACG Thr	TTT Phe	GGC Gly	AAT Asn 595	GGA Gly	GAC Asp	TGC Cys	CCT Pro	GGC Gly 600	AAT Asn	2009
GGA Gly	GAG Glu	AAA Lys	CTG Leu 605	AAG Lys	AAA Lys	TCG Ser	CTC Leu	TTC Phe 610	ATG Met	CTG Leu	AAA Lys	GAG Glu	CTC Leu 615	AAC Asn	AAC Asn	2057
AAA Lys	TTC Phe	AGG Arg 620	TAC Tyr	GCT Ala	GTG Val	TTT Phe	GGC Gly 625	CTC Leu	GGC Gly	TCC Ser	AGC Ser	ATG Met 630	TAC Tyr	CCT Pro	CGG Arg	2105
TTC Phe	TGC Cys 635	GCC Ala	TTT Phe	GCT Ala	CAT His	GAC Asp 640	ATT Ile	GAT Asp	CAG Gln	AAG Lys	CTG Leu 645	TCC Ser	CAC His	CTG Leu	GGG Gly	2153
GCC Ala 650	TCT Ser	CAG Gln	CTC Leu	ACC Thr	CCG Pro 655	ATG Met	GGA Gly	GAA Glu	GGG Gly	GAT Asp 660	GAG Glu	CTC Leu	AGT Ser	GGG Gly	CAG Gln 665	2201
GAG Glu	GAC Asp	GCC Ala	TTC Phe	CGC Arg 670	AGC Ser	TGG Trp	GCC Ala	GTG Val	CAA Gln 675	ACC Thr	TTC Phe	AAG Lys	GCA Ala	GCC Ala 680	TGT Cys	2249

FIG. 1D

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GAG Glu	ACG Thr	TTT Phe	GAT Asp 685	GTC Val	CGA Arg	GGC Gly	AAA Lys	CAG Gln 690	CAC His	ATT Ile	CAG Gln	ATC Ile	CCC Pro 695	AAG Lys	CTC Leu	2297
TAC Tyr	ACC Thr	TCC Ser 700	AAT Asn	GTG Val	ACC Thr	TGG Trp	GAC Asp 705	CCG Pro	CAC His	CAC His	TAC Tyr	AGG Arg 710	CTC Leu	GTG Val	CAG Gln	2345
GAC Asp	TCA Ser 715	CAG Gln	CCT Pro	TTG Leu	GAC Asp	CTC Leu 720	AGC Ser	AAA Lys	GCC Ala	CTC Leu	AGC Ser 725	AGC Ser	ATG Met	CAT His	GCC Ala	2393
AAG Lys 730	AAC Asn	GTG Val	TTC Phe	ACC Thr	ATG Met 735	AGG Arg	CTC Leu	AAA Lys	TCT Ser	CGG Arg 740	CAG Gln	AAT Asn	CTA Leu	CAA Gln	AGT Ser 745	2441
CCG Pro	ACA Thr	TCC Ser	AGC Ser	CGT Arg 750	GCC Ala	ACC Thr	ATC Ile	CTG Leu	GTG Val 755	GAA Glu	CTC Leu	TCC Ser	TGT Cys	GAG Glu 760	GAT Asp	2489
GGC Gly	CAA Gln	GGC Gly	CTG Leu 765	AAC Asn	TAC Tyr	CTG Leu	CCG Pro	GGG Gly 770	GAG Glu	CAC His	CTT Leu	GGG Gly	GTT Val 775	TGC Cys	CCA Pro	2537
GGC Gly	AAC Asn	CAG Gln 780	CCG Pro	GCC Ala	CTG Leu	GTC Val	CAA Gln 785	GGC Gly	ATC Ile	CTG Leu	GAG Glu	CGA Arg 790	GTG Val	GTG Val	GAT Asp	2585
GGC Gly	CCC Pro 795	ACA Thr	CCC Pro	CAC His	CAG Gln	ACA Thr 800	GTG Val	CGC Arg	CTG Leu	GAG Glu	GAC Asp 805	CTG Leu	GAT Asp	GAG Glu	AGT Ser	2633
GGC Gly 810	AGC Ser	TAC Tyr	TGG Trp	GTC Val	AGT Ser 815	GAC Asp	AAG Lys	AGG Arg	CTG Leu	CCC Pro 820	CCC Pro	TGC Cys	TCA Ser	CTC Leu	AGC Ser 825	2681
CAG Gln	GCC Ala	CTC Leu	ACC Thr	TAC Tyr 830	TCC Ser	CCG Pro	GAC Asp	ATC Ile	ACC Thr 835	ACA Thr	CCC Pro	CCA Pro	ACC Thr	CAG Gln 840	CTG Leu	2729
CTG Leu	CTC Leu	CAA Gln	AAG Lys 845	CTG Leu	GCC Ala	CAG Gln	GTG Val	GCC Ala 850	ACA Thr	GAA Glu	GAG Glu	CCT Pro	GAG Glu 855	AGA Arg	CAG Gln	2777

FIG. 1E

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AGG Arg	CTG Leu	GAG Glu 860	GCC Ala	CTG Leu	TGC Cys	CAG Gln	CCC Pro 865	TCA Ser	GAG Glu	TAC Tyr	AGC Ser	AAG Lys 870	TGG Trp	AAG Lys	TTC Phe	2825
ACC Thr	AAC Asn 875	AGC Ser	CCC Pro	ACA Thr	TTC Phe	CTG Leu 880	GAG Glu	GTG Val	CTA Leu	GAG Glu	GAG Glu 885	TTC Phe	CCG Pro	TCC Ser	CTG Leu	2873
CGG Arg 890	GTG Val	TCT Ser	GCT Ala	GGC Gly	TTC Phe 895	CTG Leu	CTT Leu	TCC Ser	CAG Gln	CTC Leu 900	CCC Pro	ATT Ile	CTG Leu	AAG Lys	CCC Pro 905	2921
AGG Arg	TTC Phe	TAC Tyr	TCC Ser	ATC Ile 910	AGC Ser	TCC Ser	TCC Ser	CGG Arg	GAT Asp 915	CAC His	ACG Thr	CCC Pro	ACG Thr	GAG Glu 920	ATC Ile	2969
CAC His	CTG Leu	ACT Thr	GTG Val 925	GCC Ala	GTG Val	GTC Val	ACC Thr	TAC Tyr 930	CAC His	ACC Thr	GGA Gly	GAT Asp	GGC Gly 935	CAG Gln	GGT Gly	3017
CCC Pro	CTG Leu	CAC His 940	CAC His	GGT Gly	GTC Val	TGC Cys	AGC Ser 945	ACA Thr	TGG Trp	CTC Leu	AAC Asn	AGC Ser 950	CTG Leu	AAG Lys	CCC Pro	3065
CAA Gln	GAC Asp 955	CCA Pro	GTG Val	CCC Pro	TGC Cys	TTT Phe 960	GTG Val	CGG Arg	AAT Asn	GCC Ala	AGC Ser 965	GCC Ala	TTC Phe	CAC His	CTC Leu	3113
CCC Pro 970	GAG Glu	GAT Asp	CCC Pro	TCC Ser	CAT His 975	CCT Pro	TGC Cys	ATC Ile	CTC Leu	ATC Ile 980	GGG Gly	CCT Pro	GGC Gly	ACA Thr	GGC Gly 985	3161
ATC Ile	GTG Val	CCC Pro	TTC Phe	CGC Arg 990	AGT Ser	TTC Phe	TGG Trp	CAG Gln	CAA Gln 995	CGG Arg	CTC Leu	CAT His	GAC Asp	TCC Ser 1000	CAG Gln	3209
CAC His	AAG Lys	GGA Gly	GTG Val 1005	CGG Arg	GGA Gly	GGC Gly	CGC Arg	ATG Met 1010	ACC Thr	TTG Leu	GTG Val	TTT Phe	GGG Gly 1015	TGC Cys	CGC Arg	3257
CGC Arg	CCA Pro	GAT Asp 1020	GAG Glu	GAC Asp	CAC His	ATC Ile	TAC Tyr 1025	CAG Gln	GAG Glu	GAG Glu	ATG Met	CTG Leu 1030	GAG Glu	ATG Met	GCC Ala	3305
CAG Gln	AAG Lys 1035	GGG Gly	GTG Val	CTG Leu	CAT His	GCG Ala 1040	GTG Val	CAC His	ACA Thr	GCC Ala	TAT Tyr 1045	TCC Ser	CGC Arg	CTG Leu	CCT Pro	3353

FIG. 1F

SUBSTITUTE SHEET (RULE 26)

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GGC AAG CCC AAG GTC TAT GTT CAG GAC ATC CTG CGG CAG CAG CTG GCC Gly Lys Pro Lys Val Tyr Val Gln Asp Ile Leu Arg Gln Gln Leu Ala 1050 1055 1060 1065	3401
AGC GAG GTG CTC CGT GTG CTC CAC AAG GAG CCA GGC CAC CTC TAT GTT Ser Glu Val Leu Arg Val Leu His Lys Glu Pro Gly His Leu Tyr Val 1070 1075 1080	3449
TGC GGG GAT GTG CGC ATG GCC CGG GAC GTG GCC CAC ACC CTG AAG CAG Cys Gly Asp Val Arg Met Ala Arg Asp Val Ala His Thr Leu Lys Gln 1085 1090 1095	3497
CTG GTG GCT GCC AAG CTG AAA TTG AAT GAG GAG CAG GTC GAG GAC TAT Leu Val Ala Ala Lys Leu Lys Leu Asn Glu Glu Gln Val Glu Asp Tyr 1100 1105 1110	3545
TTC TTT CAG CTC AAG AGC CAG AAG CGC TAT CAC GAA GAT ATC TTC GGT Phe Phe Gln Leu Lys Ser Gln Lys Arg Tyr His Glu Asp Ile Phe Gly 1115 1120 1125	3593
GCT GTA TTT CCT TAC GAG GCG AAG AAG GAC AGG GTG GCG GTG CAG CCC Ala Val Phe Pro Tyr Glu Ala Lys Lys Asp Arg Val Ala Val Gln Pro 1130 1135 1140 1145	3641
AGC AGC CTG GAG ATG TCA GCG CTC TGAGGGCCTA CAGGAGGGGT TAAAGCTGCC Ser Ser Leu Glu Met Ser Ala Leu 1150	3695
GGCACAGAAC TTAAGGATGG AGCCAGCTCT GCATTATCTG AGGTCACAGG GCCTGGGGAG	3755
ATGGAGGAAA GTGATATCCC CCAGCCTCAA GTCTTATTTC CTCAACGTTG CTCCCCATCA	3815
AGCCCTTTAC TTGACCTCCT AACAAGTAGC ACCCTGGATT GATCGGAGCC TCCTCTCTCA	3875
AACTGGGGCC TCCCTGGTCC CTTGGAGACA AAATCTTAAA TGCCAGGCCT GGCGAGTGGG	3935
TGAAAGATGG AACTTGCTGC TGAGTGCACC ACTTCAAGTG ACCACCAGGA GGTGCTATCG	3995
CACCACTGTG TATTTAACTG CTTGTGTAC AGTTATTTAT GCCTCTGTAT TAAAAAACT	4055
AACACCCAGT CTGTTCCCCA TGGCCACTTG GGTCTTCCCT GTATGATTCC TTGATGGAGA	4115
TATTTACATG AATTGCATTT TACTTTAATC	4145

FIG. 1G

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NB 25
Exp. date
2/28/92

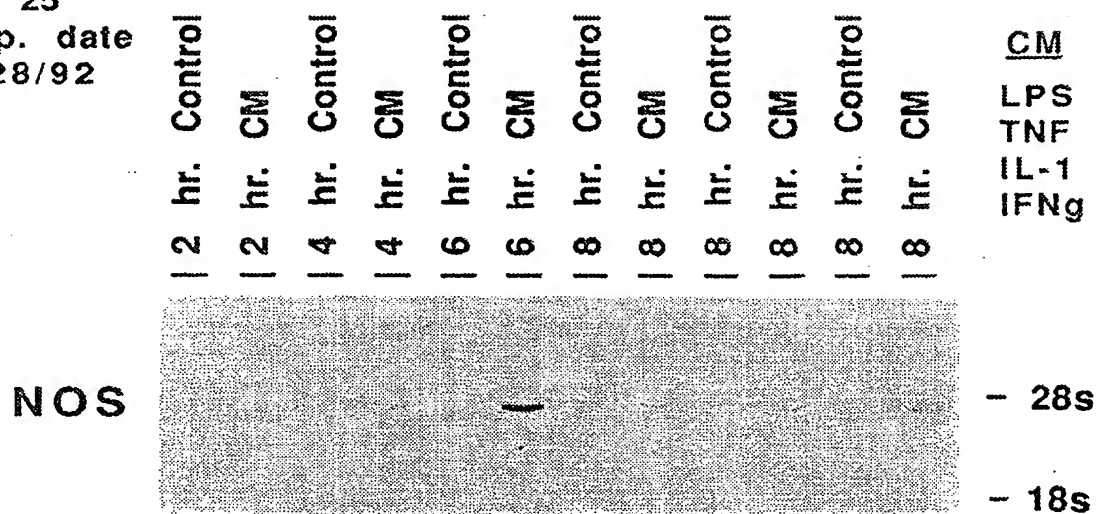


FIG. 2

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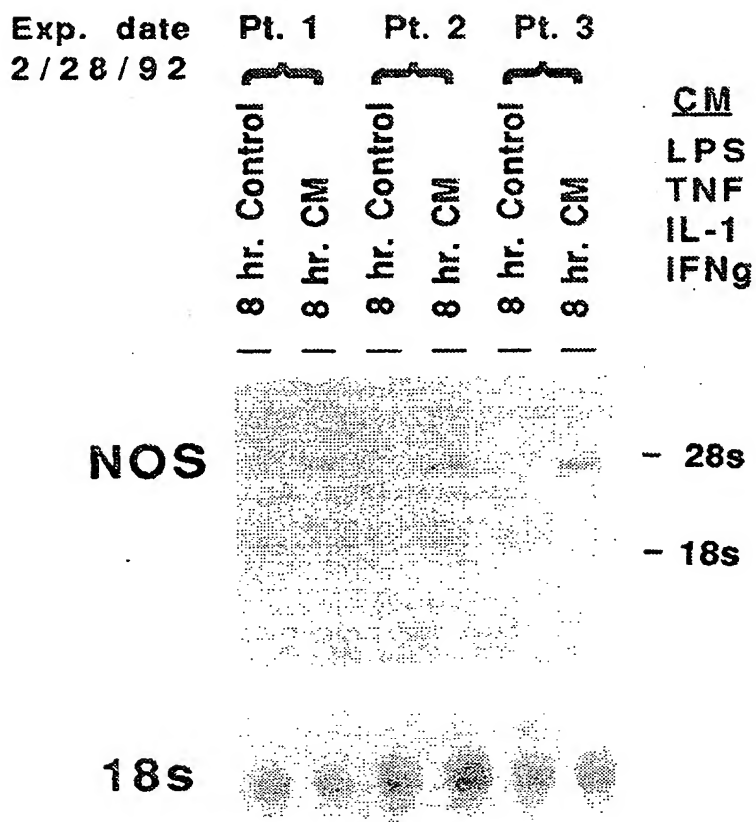


FIG. 3

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HC + CM (Pt. #1)
HC + CM (Pt. #2)

CM
LPS
TNF
IL-1
IFNg

NOS - 28s

- 18s

FIG. 4

2 hr. control	2 hr. CM	4 hr. control	4 hr. CM	6 hr. control	6 hr. CM	8 hr. control	8 hr. CM	24 hr. control	24 hr. CM	48 hr. control	48 hr. CM	CM
												LPS
												TNF
												IL-1
												IFNg

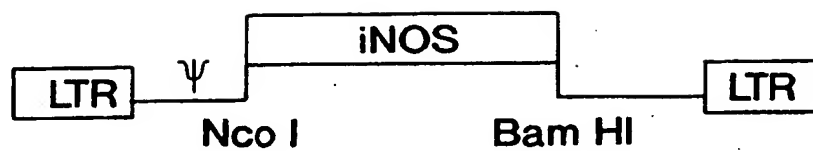
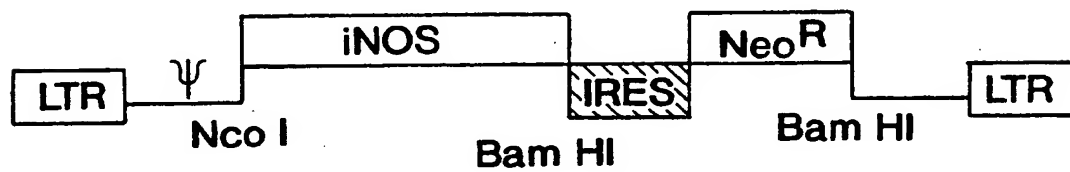
NOS

- 28s

- 18s

FIG. 5

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MFG-iNOS:DFG-iNOS-Neo:**FIG. 6**

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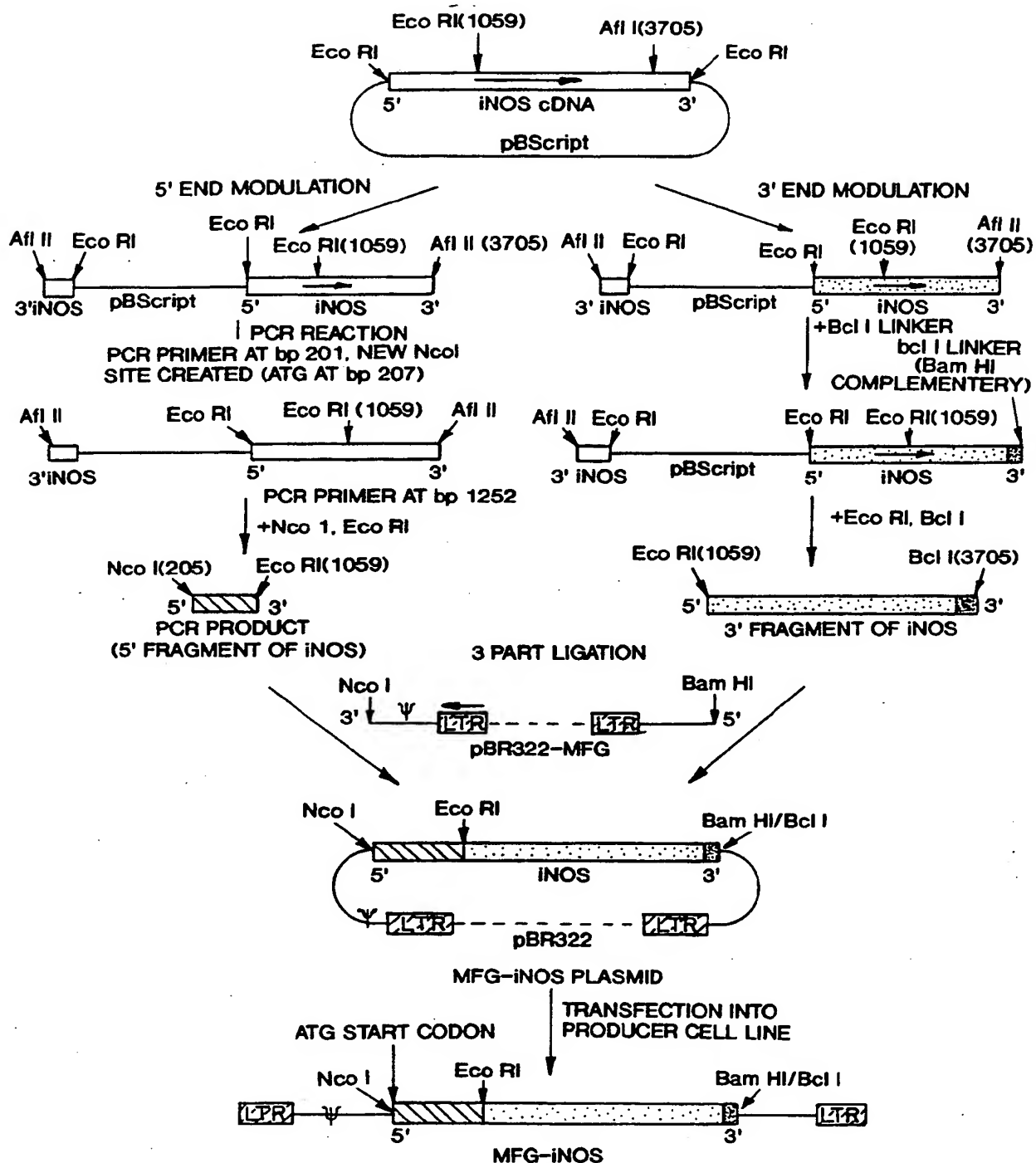


FIG. 7

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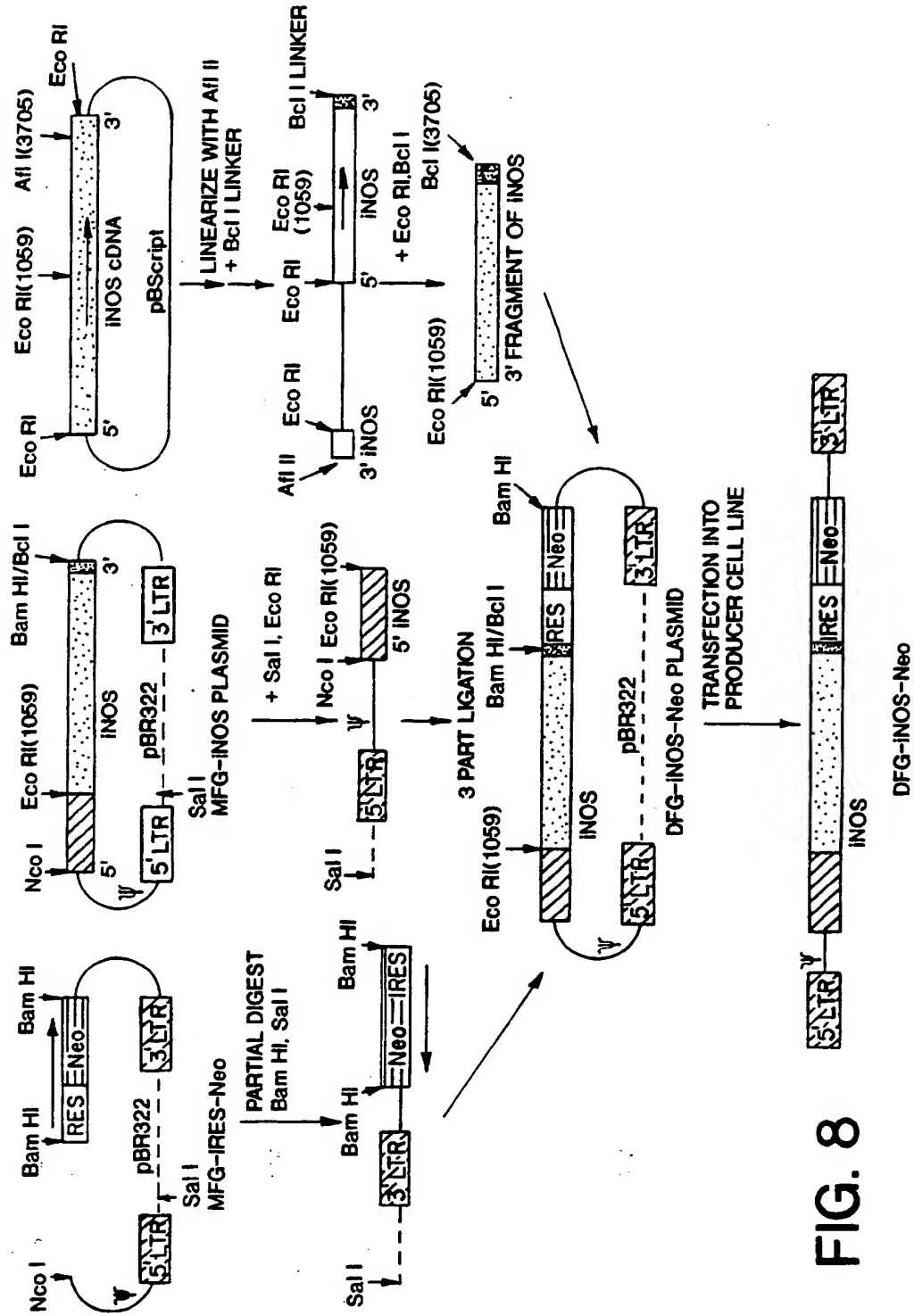


FIG. 8

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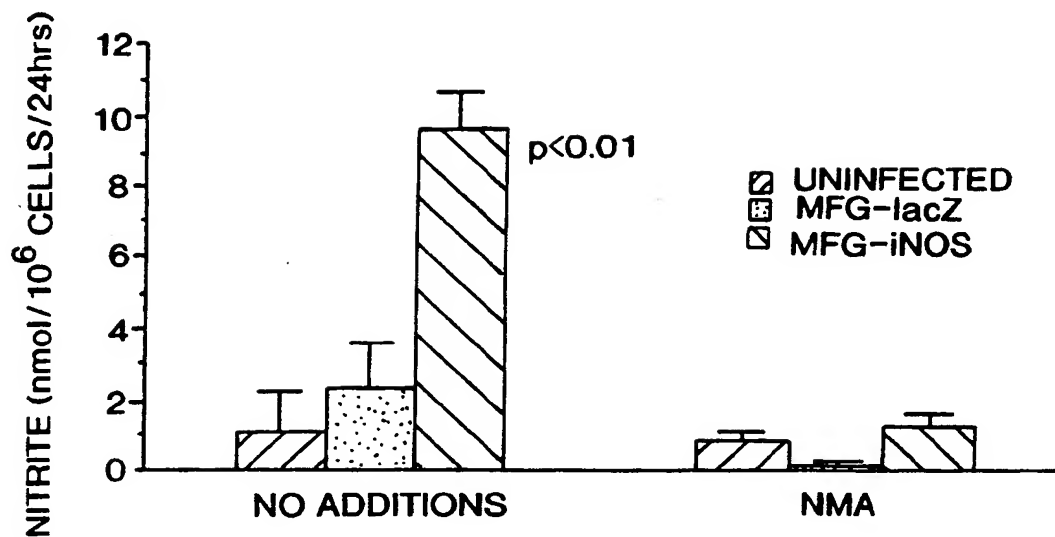


FIG. 9

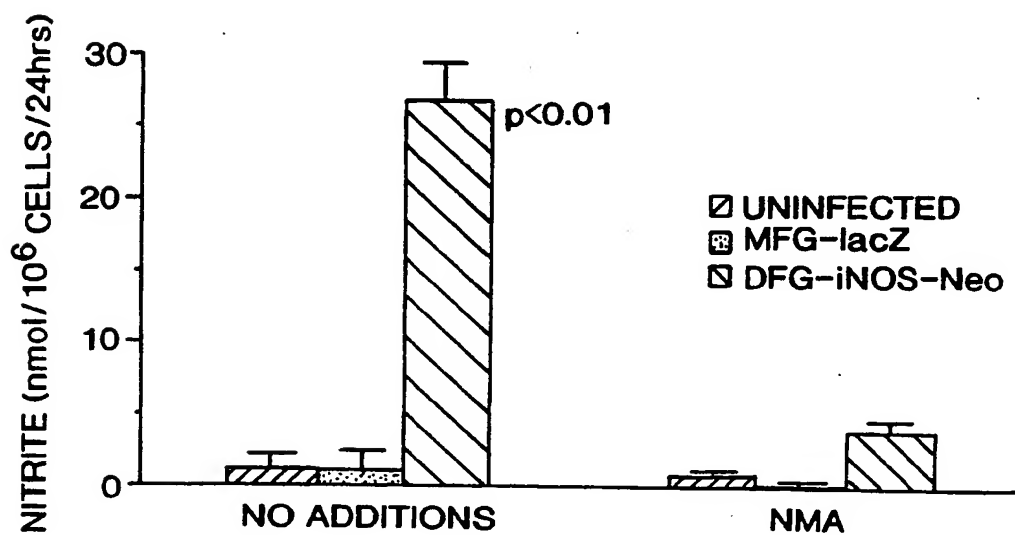


FIG. 10

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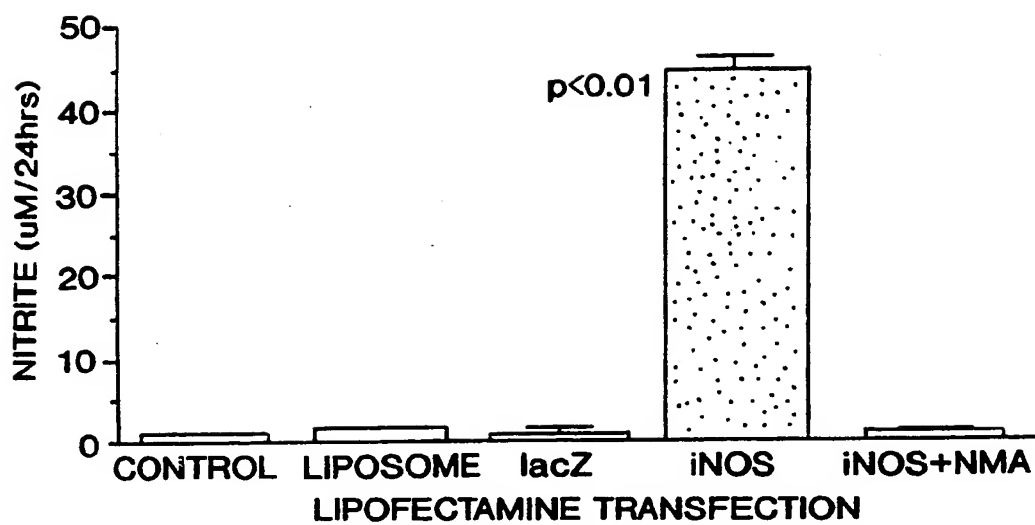


FIG. 11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/07849

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : 514/44; 424/93.2; 536/23.2; 435/172.3; 320.1, 240.2, 191

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514 / 44; 424 / 93.2; 536 / 23.2; 435 / 172.3; 320.1, 240.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proc. Natl. Acad. Sci., USA, Volume 90, issued April 1993, Geller et al., "Molecular Cloning And Expression Of Inducible Nitric Oxide Synthase From Human Hepatocytes", pages 3491-3495, see entire document.	1-77
P, Y	US, A, 5,428,070 (COOKE ET AL.) 27 June 1995, see entire document.	1-77
P, Y	US, A, 5,328,470 (NABEL ET AL.) 12 July 1994, see entire document.	1-77
Y	US, A, 5,252,479 (SRIVASTAVA) 12 October 1993	1-77
Y	US, A, 5,304,121 (SAHATJIAN) 19 April 1994	1-77

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* Inter document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

06 AUGUST 1995

Date of mailing of the international search report

11 OCT 1995

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
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Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

CHRISTOPHER S. F. LOW

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/07849

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FASEB J., Volume 8, issued 18 March 1994, von der Leven et al., "In Vivo Gene Transfer To Prevent Neointima Hyperplasia After Vascular Injury: Effect Of Overexpression Of Constitutive Nitric Oxide Synthase", page A802, abstract number 4651, see entire document.	1-77
Y	Current Topics in Microbiol. Immunol., Volume 158, issued 1992, Miller, A. D., "Retroviral Vectors", pages 1-24, see entire document.	1-77
Y	Proc. Natl. Acad. Sci., USA, Volume 89, issued December 1992, Stribling et al., "Aerosol Gene Delivery In Vivo", pages 11277-11281, see entire document.	1-77
Y	WO, A, 92/07943 (SOMATIX THERAPY CORPORATION) 14 May 1992, see entire document.	1-77
Y	WO, A, 92/07573, (SOMATIX THERAPY CORPORATION) 14 May 1992, see entire document.	1-77
Y	US, A, 4,840,940 (SOTTIURAI) 20 June 1989, see entire document.	1-77

Form PCT/ISA/210 (continuation of second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/07849

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐
☐

- The additional search fees were accompanied by the applicant's protest.
No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/07849

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

A01N 43/04; A61K 31/70; A01N 63/00; A61K 48/00; C07H 21/04; C12N 15/09, 15/63, 15/85, 5/10; C12N 9/06

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

Automated Patent System - USPAT and JPOABS
DIALOG - files 5, 50, 149, 155, 159, 357, 159, 399, 73,
GENBANK, EMBL, UEMBL

Search terms: nitric oxide synthase, DNA, gene, oligonucleotide, pCIS, plasmid, vector, mfg-iNOS, dfg-iNOS

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-31 and 61-77, drawn to polynucleotides and a process where the polynucleotides are administered in situ (cells not removed from the body) prior to transformation and are for example searched in Class 514, subclass 44

Group II, claims 32-60, drawn to a second process of using the polynucleotides where the polynucleotides are administered in vitro (cells removed from the body) prior to transformation and then implanted and are for example searched in Class 424, subclass 93.2

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons. The technical feature of transformation and selection prior to implantation is not required of the claims in Group I. Thus, the special technical features of the processes of use differ.

